

# Synthesis of biodegradable polymers for delivery of diagnostic agents

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# SYNTHESIS OF BIODEGRADABLE POLYMERS FOR DELIVERY OF DIAGNOSTIC AGENTS

Submitted by

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The research work contained within this thesis has been carried out in the School of Pharmacy and Pharmacology, under the supervision of Dr Michael D. Threadgill and Dr Colin W. Pouton.



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# ABSTRACT

Macromolecules have been investigated extensively as carriers of drugs for site-specific drug delivery. The specialised structure of tumours leads to selective accumulation of some polymers. Attachment of chelated paramagnetic ions to water-soluble polymers enhances the relaxivity of the ions; this effect, coupled with tumour tropism, makes macromolecular MRI contrast agents highly attractive.

The principal mode of uptake of macromolecules into cells is pinocytosis. Their fate is to be transported to lysosomes. If the polymer is non-degradable by the lysosomal enzymes, it will remain in the body indefinitely. Enzymically degradable polymers, therefore, represent interesting opportunities for the design of macromolecular pro-drugs and imaging agents.

A series of peptide-based monomers have been prepared using Boc- and Z-protected amino acid active esters in standard solution phase couplings. All have a sequence inverting unit (ethane-1,2-diamine) to present secondary amines at the C and N termini. Both potentially degradable and non-degradable peptide monomers have been synthesised, the former containing the sequence GlyPheLeuGly. A number also incorporate one or more side-chain functionalised amino acids to allow attachment of the potential magnetic resonance contrast agent chlorotetraphenylporphyrinato-manganese (III).

An efficient preparation of α,ω-diglycidyl ethers of poly(ethylene glycols) has been developed to provide suitable bis-electrophiles for co-polymerisation with the α,ω-bis-nucleophilic peptide monomers. Preliminary investigations of these polymerisation reactions have indicated formation of small molecular weight polymers. In one case using the 1,9-bis(sarcosylamide) of nonane-1,9-diamine as a model for the peptide, 1 : 1 macrocycles were formed. Early studies of metallation of porphyrins indicated insertion of manganese (III).

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*No man committed a greater evil*
*than he who did nothing,*
*for he could only do a*
*little.*

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## SCHEMES

# ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin Converting Enzyme |
| AIBN | α,α'-Azobisisobutyronitrile |
| Boc | t-Butoxycarbonyl |
| BOPTA | Benzyloxypropionatotetraacetic Acid |
| CDI | 1,1'-Carbonyldiimidazole |
| COSY | Correlation Spectroscopy |
| DCC | N,N'-Dicyclohexylcarbodiimide |
| DCU | N,N'-Dicyclohexylurea |
| DDQ | 2,3-Dichloro-5,6-dicyanobenzoquinone |
| DMAP | 4-(Dimethylamino)pyridine |
| DMF | Dimethylformamide |
| DNA | Deoxyribonucleic Acid |
| DOTA | TetraazadodecanetetraaceticAcid |
| DPDP | N,N'-Dipyridoxalethylenediamine-N,N'-diacetate-5,5'-bis(phosphate) |
| DTPA | Diethylenetriaminepentaacetic Acid |
| DTPA-SA | Diethylenetriaminepentaacetic Acid Stearyl Amide |
| EDC | 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide Hydrochloride |
| EDTA | Ethylenediaminetetraacetic Acid |
| EEDQ | N-Ethoxy-1,2-dihydroquinolone |
| EHPG | Ethylenebis(2-hydroxyphenylglycine) |
| EPC | Egg Phosphatidyl Choline |
| EPR | Enhanced Permeation and Retention |
| FAB | Fast Atom Bombardment |
| FMOC | Fluoren-9-ylmethoxycarbonyl |
| GPC | Gel Permeation Chromatography |
| HOBT | 1-Hydroxybenzotriazole |
| HPMA | Hydroxypropylmethacrylamide |
| LDH | Lactate Dehydrogenase |
| LDL | Low Density Lipoprotein |
| MDP | N-Acetylmuramyl Dipeptide |

| | |
|---|---|
| MMC | Mitomycin C |
| MRI | Magnetic Resonance Imaging |
| MS | Mass Spectrometry |
| α-MSH | α-Melanocyte Stimulating Hormone |
| NAp | *p*-Nitroaniline |
| NMR | Nuclear Magnetic Resonance |
| NOTA | 1,4,7-Triazacyclononanetriacetate |
| NP | 4-Nitrophenyl |
| NSu | Succinimido |
| PEG | Poly(ethylene glycol) |
| PFP | Pentafluorophenyl |
| PHEA | Poly(α,β-(N-2-hydroxyethyl))-D,L-aspartamide |
| PHEG | Poly(hydroxyethylglutamate) |
| PHG | Poly(glutaminylhistamine-glutamate) |
| PVP | Polyvinylpyrrolidone |
| RT | Ambient Temperature |
| SA | Stearyl Amide |
| SE | Stearyl Ester |
| TCP | 2,4,5-Trichlorophenyl |
| TES | N-Succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl |
| TFA | Trifluoroacetic Acid |
| THF | Tetrahydrofuran |
| TLC | Thin Layer Chromatography |
| TPP | 5,10,15,20-Tetraphenyl-21*H*,23*H*-porphine |
| TPPS | 5,10,15,20-Tetra(4-sulphonatophenyl)-21*H*,23*H*-porphine |
| Troc | 2,2,2-Trichloroethoxycarbonyl |
| UV | Ultra-Violet |
| VPM | Vascular Permeability Mediator |
| WSCI | Water-Soluble Carbodiimide |
| Z | Benzyloxycarbonyl |

# CHAPTER ONE

## ACCUMULATION OF MACROMOLECULES IN TUMOUR TISSUES

> "I did as physicians do when they, in order to make a medicament for the benefit of the liver, add sugar, honey or some other sweet stuff, because the liver likes sweet things. And due to this preference for the sweet, the liver attracts the sweet and the medicament with it. The same is done with every organ which needs a medicament: the drug is mixed with something the organ naturally is inclined to." [1]

### 1.1 Introduction

The dream of site-specific drug delivery has existed for many years, as can be seen from this 14th Century quotation, but has still to reach a reality in the treatment of many diseases, especially cancer. Paul Ehrlich, first proposed the modern form of site-specific drug delivery, with the 'magic bullet' approach; the targeting of drugs to cells *via* receptors [2,3].

The most simple methods of site-specific drug delivery involve either direct treatment of diseases of the nasal, vaginal and anal passages or implantation of solid devices to the affected area, allowing controlled release of a drug. Both these approaches are unsuitable for the treatment of most forms of cancer, as the tumour is often inaccessible and / or diffuse.

More recently, a general approach to site-specific drug delivery using carriers has been investigated. These carriers protect the drug from degradation prior to interaction with the target cells but, more importantly, protect the body from the toxic effects of the drug [4]. By incorporation of targeting moieties to interact with cellular receptors, site-specific drug delivery can be achieved. Many potential carriers have been investigated including red blood cells [3,4], low density lipoprotein (LDL) [4] and antibodies [5]. Limited success has been achieved with these carriers; they are, on the whole, difficult to manipulate and characterise. More useful approaches based on synthetic polymers and lipids have been more widely investigated [2,6].

Liposomes are formed from polar phospholipids which, in contact with water, form concentric lipid bilayers. The aqueous core thus formed can be used to carry water-soluble drugs, whereas hydrophobic drugs can be incorporated into the lipid. Liposomes are very versatile and, by careful choice of lipid, the size, number of lamellae, charge and stability can be altered [4]. These modifications provide variations in the pharmacokinetics of the liposomes and allow targeting to organs [6]. Investigators have also incorporated specific targeting residues such as sugars to evaluate the possibility of specific drug targeting [2]. However, despite such attempts, because of their size most liposomes are taken up by the particle recognition systems of reticulo-endothelial cells. Thus, at present, few liposomal medicines are available. However, a liposomal formulation of the antifungal drug amphotericin B is available, which shows reduced toxicity, thus allowing increased dosages [7].

Another approach to site-specific drug delivery is the use of microspheres or nanoparticles [8]. These are small polymer particles which either encapsulate a drug or contain drug in the matrix. Again, because of their size, they are prone to capture by the reticulo-endothelial system, although targeting moieties can be incorporated. By appropriate choice of the polymer, it is possible to provide a system that only degrades in the desired region and hence selectively releases drug. This targeting can be achieved on two levels, either to a specific area of the body (for example, the use of polymers which degrade in the anaerobic conditions of the colon), or within a cell (for example, by using acid-sensitive linkages).

The most useful approach to site-specific drug delivery is that of macromolecular pro-drugs. These were first proposed by Ringsdorf in 1975 [6,9] and an idealised model of such a system is shown in Figure 1.1 By attaching a drug of low molecular weight to a macromolecule, two main effects can be achieved: firstly, a controlled release system and, secondly, a targeted system. The macromolecule itself should be non-toxic and non-immunogenic and should be easily manipulated for chemical reactivity [2]. Originally, the macromolecules used were naturally occurring, for example DNA [10], albumin [11] and dextran [12]. However, synthetic polymers, which have been previously used as plasma expanders, are now more commonly used [2]. Targeting groups can be easily attached to the polymer and these can either increase non-specific cellular interactions [13], or exploit receptor interactions to elicit a specific effect [14]. Drug molecules can either be attached to the polymer *via* a non degradable spacer or by degradable linkages [15,16].

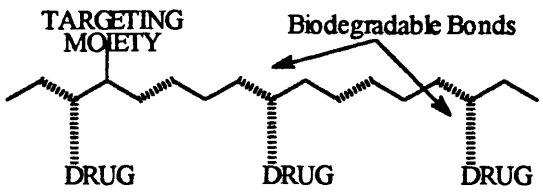



Figure 1.1

Attachment of a drug to a macromolecule is of particular interest as a therapy for cancer. Macromolecules are known to accumulate within solid tumours, giving a first level of targeting [17]. This accumulation can be enhanced by incorporation of specific targeting groups. Once a drug is attached to a macromolecule, it can no longer enter a cell by diffusion and must enter by endocytosis which is often enhanced in tumour cells [2]. Furthermore, once access to the cell is limited to endocytosis, 'lysosomotrophic drugs' can be produced where release of drug only occurs within the lysosomes, thereby conferring a high level of targeting [10].

## 1.2 Uptake of macromolecules into tissues

Many investigators, have demonstrated an increased uptake of macromolecules into tumour cells [18,19,20]. Originally, this was thought to be due to an increase in the rate of constitutive pinocytosis in tumour cells [18]. However, more recent studies have suggested that the increased uptake may be due to an "Enhanced Permeation and Retention Effect" (EPR effect) [17].

Matsumura and Maeda [17] investigated the accumulation in tumours of a series of radioactive macromolecules and Evans' blue, a dye. The uptake of a range of polymers of molecular weight 12,000 Da to 150,000 Da was investigated in tumour bearing mice with respect to the plasma clearance time and the time to reach a fixed gradient (5 :1) between the tumour and plasma. Macromolecules of above 16,000 Da accumulated in the tumours, all reaching the desired ratio. However, it was apparent from the data that the ideal range for the macromolecules was between 15,000 Da and 70,000 Da.

The investigators proposed that the accumulation was due to four properties of tumour tissues; increased angiogenesis, enhanced vascular permeability, little macromolecular recovery *via* post capillary venules and lack of functioning lymphatic systems [17].

### 1.2.1 Angiogenesis in Tumours

The rate of angiogenesis in some tumour tissues is greatly increased [19,20]. Tumour growth is dependent on the development of a new functioning vascular system. To achieve this increased growth, tumours secrete a range of angiogenic factors, such as the polypeptide angiogenin [17,21]. These factors either act directly on the dividing cells or stimulate the realise of endothelial growth factors from other cells. The increased number of blood vessels can be easily demonstrated using angiography [17].

### 1.2.2 Enhanced Vascular Permeability

#### 1.2.2.1 Capillary Types

There are three main types of endothelial capillaries; continuous, fenestrated and sinusoidal [1]. In all of these, the endothelial cells are similar. Each cell has approximate dimensions of 20-40 μm long, 10-15 μm wide and 0.1-0.5 μm thick [22]. These form a single layer above a basement membrane and are covered by a layer of glucosoaminoglycans [22]. However, in each organ, the endothelium is highly distinctive [22].

The three types of endothelium are illustrated in Figure 1.2 . Continuous capillaries are seen in normal connective tissue and the blood brain barrier [22]. They are the least permeable to all molecules but especially to macromolecules, having tight junctions between the cells of approximately 2 nm. Post-capillary venules are a slightly modified type of continuous capillaries. Here the junctions are larger (≅6 nm), allowing easier passage of some molecules [19].

Fenestrated capillaries have an intact basement membrane, but in some places have large interendothelial junctions of 40-60 nm (fenestrae). The fenestrations can be open, as in the case of the glomerulus, or closed by thin diaphragms [19,22]. These capillaries are mainly found in the kidney, endocrine and exocrine glands. They are also found in some tumours [19].

Sinusoidal capillaries are found in only a few organs, most notably the liver and spleen, and in some tumours. These capillaries allow easy access of molecules to the interstitium, as they often exhibit a lack of, or a discontinuous, basement membrane and large interendothelial spaces (150 nm) [19].

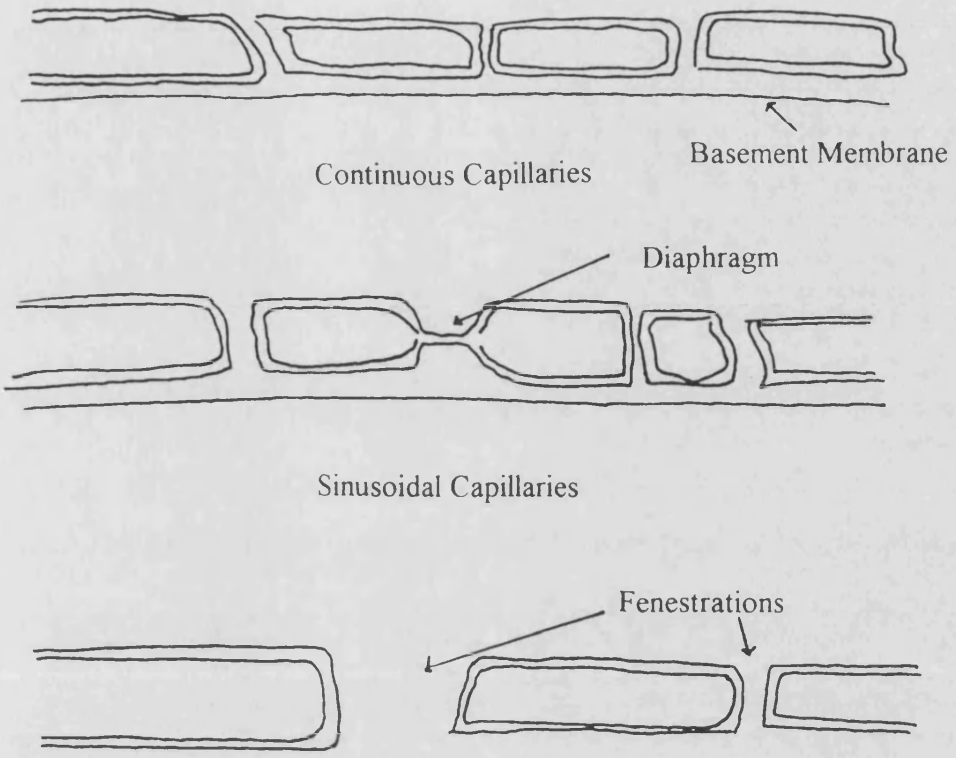



Figure 1.2 : Types of Capillary

1.2.2.2 Transport of molecules across capillary endothelium

Five types of molecular movement through endothelia have been described [22]:

1. Simple diffusion of the molecule across the cell
2. Lateral diffusion of the molecule in the cell membrane allowing transport around the cell and across the barrier
3. Through inter-endothelial cell junctions
4. Through endothelial gaps *i.e.* fenestrae of sinusoids
5. Vesicular transport

The first two types can be discounted when considering the transport of macromolecules, although they are the main methods of transport for small solutes. All of the other types are involved in the transport of macromolecules. The majority of transport is thought to be based on the formation of water filled pores at the endothelial junctions [22]. The role of vesicles in transport is more widely disputed [19,22]. Vesicular transport can take two forms; the uptake of a macromolecule into a vesicle followed by transport across the cytoplasm and release, or linkage of vesicles to form a transient pore across the cell. The importance of the first method is questionable; some workers have suggested it to be the major method of transport of macromolecules, whereas other feel its role is minimal. The latter hypothesis is supported by the observations of no change in the rate of transport at reduced temperature where vesicular transport is known to be abolished [19,22].

The transport of macromolecules can be affected by both their size and charge. Measurement of the glomerular filtration rate, an example of endothelial transport, has demonstrated that the hydrodynamic radius of a macromolecule is a more accurate description of the tendency to be transported than is molecular weight [17]. Hence, it has been proposed that albumin, a natural macromolecule, has a small radius, due to strong hydrogen bonding, and therefore a large renal threshold, whereas a hydrophobic synthetic polymer with few intramolecular bonds, such as HPMA, shows lower renal thresholds [19]

The charge of a macromolecule can also affect transport across endothelial cells. The endothelial surface exhibits a small negative charge and experiments with albumin have shown that cationic albumin is three times more permeable than neutral albumin [19].

1.2.2.3 Tumour Endothelium

Most tumours are epithelial and have vasculature distinct from that of normal tissue. The endothelium, in all but a few cases, is continuous having tight endothelial junctions and an intact basement membrane, unlike inflammatory tissue. However, as

accumulation of macromolecules occurs, the endothelium must be more permeable than normal continuous endothelium [19].

In a very few tumours, angiogenesis is altered and the new blood vessels produced are composed of only endothelial cells, whereas most vessels also contain pericytes. Pericytes are thought to be involved in the maturation of vessels and it has been postulated that lack of pericytes can lead to incomplete basement membranes. This alteration in basement membrane could lead to a non-specific 'leakiness' of cells, allowing accumulation of macromolecules [19].

However, in the majority of cases, no morphological differences in the blood vessels can be observed. This has lead to the hypothesis that tumour cells secrete factors which alter the permeability of endothelium to macromolecules [19].

Senger *et al.* [23] have investigated the uptake of labelled human serum albumin and colloidal carbon in tumour bearing guinea pigs. Accumulation was demonstrated in the regions around the tumour and within the tumour. Analysis of the ascitic fluid from the animals and tissue culture experiments allowed the isolation of a protein which has been called the vascular permeability mediator (VPM). The isolated factor caused increased permeability in cutaneous tissues without causing endothelial cell damage [19]. This protein of between 34,000 and 42,000 Da has been isolated from a range of tumour cells [24]. These workers have suggested that this factor is released to allow the passage of fibrin into the interstitium to enable coagulation, which is beneficial to tumour growth and angiogenesis [25].

A second factor, bradykinin, has also been investigated as a promoter of tumour permeability [26]. Increased concentrations of both bradykinin and $Hyp^3$-bradykinin have been found in numerous tumour types including stomach, pancreatic and ovarian cancer [20,26]. Bradykinin is known to have both permeability and pain-inducing properties but it also is involved in the activation of phospholipase-$A_2$, leading to the formation of another permeability factor, prostaglandin $E_2$.

Other permeability factors have been investigated. Obvious candidates are the leukotrienes, which have been found in increased levels in most inflamed tissue but not, as yet, in tumour cells. Other potential permeability enhancers include, tumour necrosis factor, platelet derived growth factor, serotonin, interleukin 2 and leukokinin [20,26].

### 1.2.3 Macromolecular Recycling

Once molecules are present in the interstitium, they can be taken into cells of the organ or passed back into the blood stream. Small molecules generally pass easily into the blood stream *via* the post capillary venules, as described earlier. The endothelial cells of these contain slightly enlarged interendothelial junctions. However, these spaces are too small for the passage of macromolecules [19].

Natural macromolecules [19,20,24,26] pass into lymph capillaries. These vessels are formed from capillaries but have very large endothelial spaces. These capillaries form a separate circulation allowing the movement of macromolecules away from an organ. The macromolecules are eventually passed from the lymphatic system into the thoracic duct which empties into the blood stream *via* the sub-clavian vein [17,19].

Macromolecules of lipid nature are particularly attracted into the lymphatic system. Experiments [20] involving Lipiodol, an iodinated derivative of poppy seed oil, have shown that it is selectively taken into lymphatics and 'recycled' via the lymphatic system. When Lipiodol was injected into a tumour-feeding artery [26]; however, it accumulated in the tumour. This suggests that tumours have little lymphatic drainage [27]. This has been confirmed by imaging experiments which have shown decreased lymphatic development in tumours [17,19]. Macromolecules in tumour tissues can therefore only escape by slowly diffusing through the tumour mass to the surface where they can be recovered by the well-developed lymphatic system of surrounding organs. This transport is minimal, as macromolecules are too large to diffuse easily. This results in accumulation of the macromolecule and the formation of a depot of 'drug' [19].

This combination of enhanced permeability and lack of lymphatic drainage results in an highly effective "passive" targeting of macromolecules and hence macromolecular pro-drugs to tumour cells. The more specific 'active' targeting of cells to particular cell lines will be considered later.

### 1.2.4. Enhancement of the Enhanced Permeability and Retention Effect

Following the identification of the EPR effect, Maeda [20,26] has investigated ways of manipulating the properties which lead to enhanced uptake, to allow even more effective chemotherapy using macromolecular pro-drugs.

#### 1.2.4.1 Enhancement of Vascular Permeability

As discussed earlier, bradykinin, exhibits an effect on the vascular permeability either directly or through prostaglandin-$E_2$ [26]. It is formed by the action of kallikrien on kininogen and is continually broken down by kinase I and II enzymes (Figure 1.3). Inhibition of kinase I and II enzymes will lead to increased levels of bradykinin and hence to increased vascular permeability. These enzymes are inhibited by inhibitors of angiotensin converting enzymes (A.C.E.), for example, captopril and enalapril [20]. Concurrent administration of an inhibitor with a macromolecule would lead to enhanced uptake of the macromolecule into the tumour, whilst normal tissue would remain unaffected as the kinin cascade would not be in operation. Investigations on the uptake of labelled albumin into a mouse ascitic tumour in the presence of such an inhibitor (10 mg $kg^{-1}$ enalapril) showed a 50% increase [20].

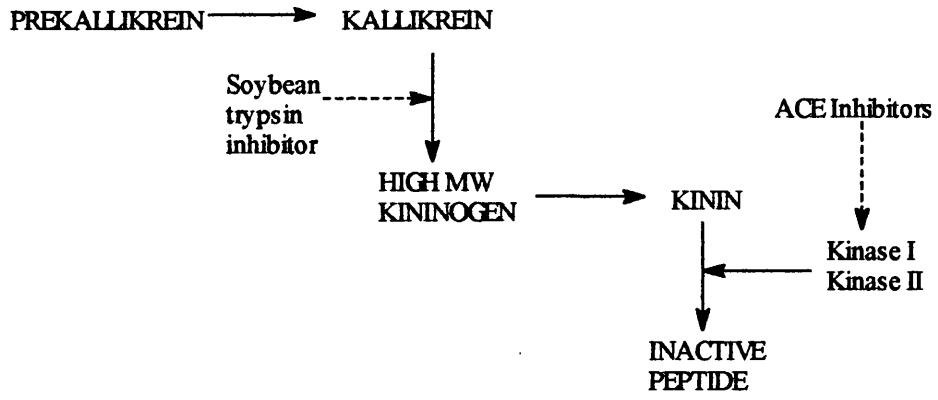



Figure 1.3

In a few tumours, this exploitation could prove disastrous as macromolecular influx also results in fluid accumulation which can be difficult to control. In this case, the administration of soy-bean trypsin inhibitor, which halts the cascade, would be more beneficial [20].

1.2.4.2 Enhancing Vascular Variations

As discussed earlier, many tumours have a hypervasculature allowing increased blood flow to the tumour. These blood vessels have been shown to be unaffected by angiotensin II. This is thought to be due to a lack of contractile smooth muscle in the blood vessels rather than lack of receptors for angiotensin II [26]. Thus, if a hypotensive state was to be produced in a patient, the junctions between the endothelium would be tightened and less passage of macromolecules would occur. However, in tumour vasculature, the vessels would remain as normal, allowing passage of macromolecules [26].

Clinical data have been produced using this method with small molecules. Both an increased regression of tumour size and improved response rate was demonstrated [26]. It is thought that with small molecules once the hypertension wears off, back flow of the molecules can occur, this would not be the case with macromolecules. Thus, studies with macromolecules [24] have shown a two fold concentration enhancement in tumours six hours after the hypotensive event as well as decreased levels in the bone

marrow. Unfortunately, this approach is of little value in naturally hypertensive patients.

## 1.3 Uptake of Macromolecules into Tumour Cells

Small solutes are easily taken into cells, *via* passive diffusion or through the action of specific porters for certain substrates such as glucose and amino acids [28]. Macromolecules, however, cannot enter a cell by such a route because of their size. Natural macromolecules such as proteins, polysaccharides and polynucleotides are taken into the cell by endocytosis. Synthetic macromolecules also follow this route [28].

Endocytosis can be considered to be of two types; pinocytosis or phagocytosis. Phagocytosis or 'eating' occurs only in specialised cells such as macrophages [29] and is the uptake of particles larger than 10-20 μm in diameter. It is of major interest in the immune system, as it is involved in the uptake and destruction of bacteria [29]. This process is of minor importance when considering soluble macromolecular pro-drugs but can be significant in approaches to drug targeting using liposomes and microparticles. Both types of endocytosis follow the same essential steps and these will be described in respect to pinocytosis.

### 1.3.1 Pinocytosis

Whereas phagocytosis occurs only in response to an event [30], pinocytosis is a constitutive phenomenon in all mammalian cells [29]. It has been likened to drinking as it involves the continual uptake of extracellular fluid. Pinocytosis is also known as fluid phase endocytosis.

The initial endocytic event in pinocytosis is the formation of an invagination in the plasma membrane. A vesicle (a pinosome) is then formed encapsulating extracellular fluid. This then buds away from the cell surface into the cytoplasm. The pinosome then fuses with other pinosomes without release of the contents. This agglomeration of pinosomes then fuses with a lysosome (a vesicle from the Golgi Apparatus) to form

a secondary lysosome. Within the secondary lysosome, degradation of macromolecules occurs and small molecules are released into the cytoplasm. The secondary lysosome then forms both a residual body, which contains non-degradable material and remains in the cell for its lifetime, and vesicles which can fuse with the cell surface allowing exocytosis of the contents [3,17,28,30]. This is expressed diagramatically in Figure 1.4.

Each cell has a basic rate of pinocytosis which can be measured by the use of polymers, such as inulin, polyvinylpyrrolidone (PVP)[30] or hydroxypropylmethacryl-amide (HPMA)[31,32] which show no specific adsorption to the cell surface. In this case, uptake by fluid phase pinocytosis is directly related to the concentration of the 'probe' in the medium. The rate of uptake is expressed as the Endocytic Index - "the volume of the culture medium which is captured in a defined number of cells in a defined time" [33]. This allows comparisons to be made on the uptake of various macromolecules and on the differing rates of uptake in different cell lines. The Endocytic Index also allows the measurement of three other types of pinocytosis, non-specific adsorptive pinocytosis, receptor mediated pinocytosis and 'piggy back' endocytosis [33].

### 1.3.2 Adsorptive Pinocytosis

Measurement of the Endocytic Index for some macromolecules shows an increase over the model polymers [33]. Two hypotheses for this event have been put forward Firstly, the macromolecule is taken up by fluid phase pinocytosis but exerts a pharmacological effect on the cell leading to an increased rate of constitutive pinocytosis. This hypothesis can be refuted easily by measuring the rate of uptake of a fluid phase 'probe' in the presence of the test macromolecule. If the rate of uptake of the 'probe' is unaltered, the test macromolecule cannot have been taken up by fluid phase pinocytosis [33].

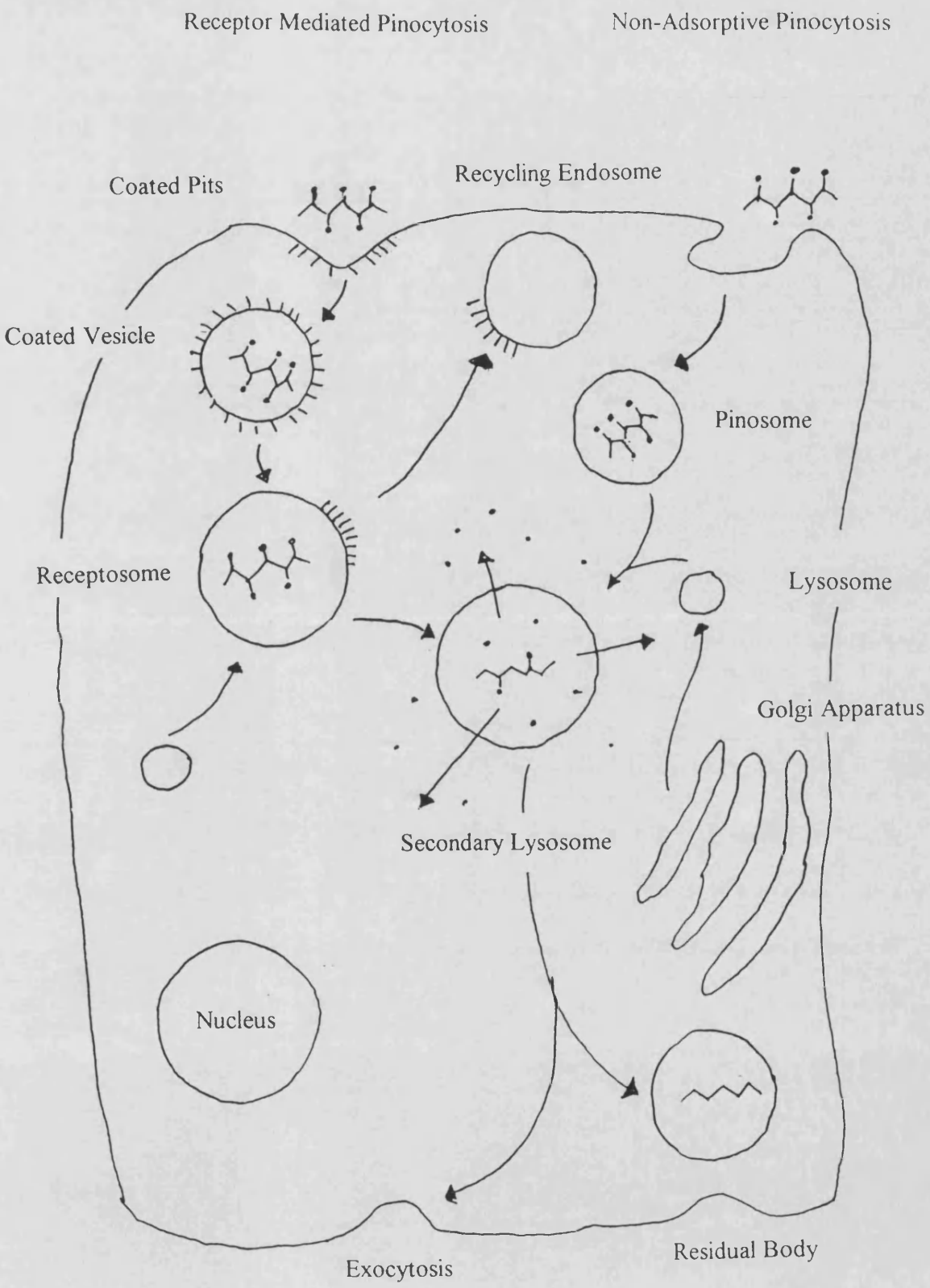



Figure 1.4 : Endocytic Pathways

The second hypothesis is more likely, that the macromolecule is internalised faster due to adsorptive endocytosis, that is a direct interaction with the cell surface resulting in the formation of a pinosome. However, it is possible that adherence can occur without pinosome formation and internalisation. This must be considered before the measured Endocytic Index can be taken as the true value. This can be easily examined by the administration of an inhibitor of endocytosis, such as 2,4-dinitrophenol [33].

There are two types of adsorptive pinocytosis; non-specific - due to changes in hydrophobicity or charge, and receptor mediated pinocytosis - where specific interactions between receptor and substrate occur [30].

1.3.2.1 Receptor Mediated Pinocytosis

The interaction of a ligand with a receptor leads to a slightly altered pinocytic pathway (Figure 1.4). Firstly, the ligand binds to receptor sites which are spread over the surface of the cell. Clustering of the receptors then occurs on the cell surface in areas called coated pits. These areas have on their interior surface a protein called clathrin. It was thought that these pits then 'bud' off to form coated vesicles which then pass the ligand for degradation whilst allowing the recycling of the receptor-clathrin complex to the cell surface *via* a recycling endosome. More recently, it has been suggested that that the clathrin does not enter the pinosome and uncoated vesicles containing receptor and ligand (receptosomes) are passed into the cell for processing [30,34].

1.3.3 'Piggy Back' Endocytosis

'Piggy Back' pinocytosis is the enhanced uptake of one molecule due to its binding to another molecule [35]. It is the process which is exploited by all macromolecular pro-drugs, the binding of a small molecule to a macromolecule restricts its uptake to endocytosis and leads to reduced non target interactions [10]. An early example of the use of 'piggy back pinocytosis' to elicit a therapeutic effect was demonstrated by Trouet *et al.* [2,6] Ethidium bromide is active against *Trypanosoma cruzi in vitro* but is

inactive *in vivo*. In host organisms, the parasite lives within lysosomes where it is unharmed by lysosomal enzymes. It is protected from ethidium bromide, since this is a small molecule which passes into cells by diffusion and cannot pass into lysosomes. Attachment of ethidium bromide to DNA results in death of the parasite as the conjugate is taken up by pinocytosis and can act within the lysosome [2].

This process can be used to explain many early results that suggested an enhanced uptake of a macromolecule due to an increase in the rate of formation of pinosomes. For example, the uptake of colloidal radioactive gold was enhanced in the presence of poly (L-lysine). This is more likely to be due to association and the macromolecule being taken up by non-specific adsorptive pinocytosis than an increased rate of formation of pinosomes [35].

### 1.3.4. Factors affecting rate and type of pinocytic uptake

#### 1.3.4.1 Hydrophobicity and Charge

Early experimental work [33] demonstrated an increased rate of uptake of denatured bovine serum albumin over untreated albumin in rat yolk sacs. As the rate of formation of pinosomes was unchanged, these results were attributed to an increased affinity for the cell surface, leading to an increase in non-specific adsorptive pinocytosis. This increase was dependent on the extent of denaturation but was unaffected by the method of denaturation. The researchers believed that this increase was due to either the unmasking of hydrophobic groups or the masking of charged residues [33]. With other denatured proteins such as insulin the rates were either unaffected by the process or showed decreased binding. Thus it was proposed that binding sites for both hydrophobic regions and charged regions exist in yolk sacs [33]. Polypeptides are very difficult to study as each amino acid can exert an effect on the binding capabilities. Experiments with model polymers, however, give more reliable data on cell surface interactions. As has been mentioned earlier, both PVP and HPMA have no affinity for the cell surface and are taken up by fluid phase pinocytosis, hence they are good models for investigating the effect of hydrophobic groups [33].

Duncan *et al* [13] have demonstrated *in vitro* that the incorporation of tyrosinamide residues onto (HPMA) leads to an increased uptake in rat yolk sacs. The effect was dependent on the molar percentage of the hydrophobic moiety, the highest percentage incorporation (15.4 mol %), resulting in a 10 fold increase in the Endocytic Index. Interestingly the effect is not seen below a threshold level of 10 mol % and the authors suggest that this is due to a requirement for hydrophobic domains before non-specific binding occurs. Similar results have been reported using the polymer poly(α,β-(N-2-hydroxyethyl))-D,L-aspartamide (PHEA) [36,37,38,39]. Incorporation of a tyramine derivative at a range of concentrations between 1.2 and 21.9 mol % resulted in enhanced uptake above a threshold value of 10 mol %. The increase was verified to be due to a non-specific interaction as if both PHEA and PVP were administered no increase in the rate of uptake of PVP could be measured.

Obviously, pinocytosis can only be enhanced if the hydrophobic moieties are exposed and therefore available for interactions with the cell surface [35]. Studies [6,40] on a block co-polymer of poly(ethylene glycol) (PEG) and poly (L-lysine) (6:3) substituted with highly hydrophobic palmitoyl derivatives demonstrated no increase in uptake of the co-polymer over that of PEG alone. This initially seems surprising. However, further evaluation of the co-polymer revealed that, in solution, the polymer forms a micelle in which the hydrophobic groups are in the core and the less hydrophobic PEG is exposed to the cell surface. It should be noted that PEG alone does show an increased uptake in comparison with PVP, demonstrating a degree of hydrophobic non-specific interactions.

The effect of charge is more complex, both anionic and cationic polymers exhibit adsorptive pinocytosis characteristics. Co-polymerisation of a vinylamine with PVP (8 mol %) results in a cationic polymer. This has an increased pinocytic uptake in both rat macrophages and yolk sac [41]. Equally, the anionic polymer, DIVEMA pyran-co-polymer, is taken up by a non-specific adsorptive process [28].

1.3.4.2 Size and Molecular Weight

The effect of size and molecular weight on macromolecular uptake is more properly defined as the effect of the hydrodynamic radius of the polymer. This is affected by the ability of the polymer to form intra-molecular hydrogen or van der Waal's interactions. The size of the pinocytic vesicles is the main barrier to uptake and varies according to cell type. Ease of access to the cell surface also requires consideration [19].

Studies on both HPMA [35] and PVP [41] have shown the uptake to be size-dependent in the rat yolk sac. Co-polymers of PVP and vinylamine of molecular weight 120,000 Da were taken up slowly in comparison to those of molecular weight 46,000 Da. In comparisons using rat macrophages, uptake of the larger polymer was more efficient.

1.3.4.3 Specific Receptor Interactions

All the modifications above result in an increase in non-specific interactions; however, many cells also possess receptors for particular substrates on the cell surface. These receptors allow internalisation and formation of pinosome. Processing allows the recycling of the receptor for further binding and release of the desired substrate into the cell [30]. Thus, these receptor-substrate interactions can be exploited to allow the delivery of macromolecules to specific cell lines. Such receptor-substrate interactions include; carbohydrate-lectin, antibody-antigen and hormone-receptor interactions.

1.3.4.3.1 Lectins

The area of lectin-mediated drug delivery has been widely explored [3,42]. Many cells express lectins on their surface and most are limited to one cell type [3]. Substrates for lectins are, however more, diverse, although many do show structural similarities. *In vivo,* lectins bind complex oligosaccharides. Therefore, it is possible for one lectin to bind two structurally different sugars in different areas of the binding sites [3,42]. An example of this is the 'P'-selectin on endothelial cells which binds the oligosaccharide Lewis $^{x}$ which contains both galactose and fructose. Polymers containing either

galactose or fructose can also bind to the lectin [3]. As lectins bind oligosaccharides, it has been suggested recently that triantennary targeting ligands should be attached rather than single sugar molecules. Experiments with the asialoglycoprotein that binds N-acetyllactosamine have shown that binding of an oligosaccharide containing three N-acetyllactosamine antennae is higher than those containing one or two [3,42].

1.3.4.3.1.1 Hepatocyte asialoglycoprotein

This asialoglycoprotein is expressed in many but not all hepatocytes. The receptor is present on the venous face of hepatocytes, allowing for interactions with blood-borne substrates. The receptor consists of at least two sub-units each capable of binding one galactose residue, raising the possibility of multi-ligand binding [42].

Hepatocytes are valuable target cells for many diseases such as hepatitis [34]. The potential of targeting to hepatoma cells, however, is low. Most tumours in the liver are either primary hepatomas or secondary metastases of breast or colo-rectal cancer. The metastases comprise modified cells from the region of the primary tumour, so do not contain the asailoglycoprotein [42]. In primary tumours, expression of the asialoglycoprotein is sometimes retained but appears to be cell line dependent. The degree of expression is also often reduced. This suggests that organ specific delivery is possible but that tumour cell specific delivery is unlikely [34,42,43].

The asialoglycoprotein has been investigated using a number of polymers bearing galactose residues both *in vitro* and *in vivo*. Vansteenkiste [44] investigated the distribution of dextran containing one (6 mol%) or three D-galactose residues (8.5 mol %) *in vivo* in mice. Thirty minutes after injection, the triantennary dextran displayed the highest uptake into liver (71%). The polymer containing only one residue and the unmodified dextran accumulated less (43 % and 16 % respectively). The increase in the uptake of the triantennary containing polymer could be due to better binding, but other workers [42] have suggested that the effect is due to the larger mol percentage of galactose residues. Another possibility is that the polymer is being picked up by a different receptor, the Kupffer cell 'galactose particle' receptor [3,42].

HPMA co-polymers bearing galactose have been evaluated in both rats and mice [14,31,45,46,47]. The kinetics of the galactose substituted polymers demonstrated a more rapid clearance from blood and an increased uptake into the liver. In studies *in vitro* using the $HepG_2$ cell line, a human tumour cell line known to express the asialoglycoprotein receptor, a seven fold increase in uptake was observed using the galactose-containing polymer [31].

1.3.4.3.1.2 Fucose Receptor on L1210 Leukaemia Cells

Of major interest in drug targeting is the fucose receptor on L1210 mouse leukaemia cells [3,42]. This has been investigated by Ulbrich *et al.* [48] A conjugate of HPMA bearing the cytotoxic drug sarcolysin was further derivatised by the inclusion of fucosylamine. The fucosylamine derivative caused greater cytotoxicity *in vitro* than did the underivatised polymer [48].

A second HPMA co-polymer bearing daunomycin and fucosylamine has also been tested both in L1210 and CCRF cells (a human leukaemia cell line which does not have fucose receptors). Inclusion of fucosylamine enhanced the inhibitory effect of daunomycin in L1210 cells but not in CCRF cells. Similar polymers containing galactosamine rather than fucosylamine were no more effective than underivatised polymers [49]. These polymers have also been investigated *in vivo* in $DBA_2$ mice inoculated interperitoneally with L1210. In this situation, incorporation of the carbohydrate gave no significant advantage on day 1 after inoculation. After 3 days, however, the fucosylamine-containing HPMA was more effective than those polymers without. It has been suggested that this may be due to the increased number of fucose receptors arising from rapid proliferation of the tumour cells [46].

Studies *in vivo* using HPMA bearing fucosylamine and adriamycin gave similar results. Again, the conjugates containing fucosylamine were more effective than those without, especially at low doses. Unfortunately, an increased liver concentration of these polymers was seen, along with targeting to the tumour. This may be due to

other cells having fucose-recognising receptors, such as hepatocytes and macrophages [50].

Interestingly, attempts to increase cellular interactions using triantennary ligands [51] have been unsuccessful, although this may have been due to a lack of flexibility in the spacer employed.

1.3.4.3.1.3 Macrophage Receptor for Mannose

Macrophages are not an obvious target cell for chemotherapy. However, delivery of a macrophage-activating agent such as N-acetylmuramyl dipeptide (MDP) to the macrophages could result in increased phagocytic activity against tumour cells and metastatic cells in particular [3]. Many sugars interact with this receptor but are of two main classes, one based on D-mannose and one on L-galactose [42].

Studies involving both glucosamine and mannosamine HPMA glycoconjugates demonstrate little change in kinetics at low levels of substitution [42]. More highly substituted polymers were accumulated in the liver, possibly owing to interactions with Kupffer cells [52]. Derriers *et al* [3] investigated poly-L-lysine bearing both MDP and mannose-related sugars and were able to demonstrate activity in activating macrophages.

All of the studies performed so far, demonstrate the potential of lectin-targeting in enhancing chemotherapy. However, more investigation of the lectins expressed by tumour cells is required before such targeting becomes a clinical reality.

1.3.4.3.2. Antibodies

Over the past few decades, much interest has been focused on exploiting the specific antibody-antigen interaction as a means of targeting drugs to particular cells [2]. This approach, however, has been beset with difficulties such as immunogenicity [53]. More importantly, the realisation that tumours are formed from more than one type of cell

and thus display more than one type of hapten, has led to a reduced interest in antibody targeting [54]. Using only one antibody conjugate would not allow interactions with every tumour cell.

Despite these problems, a number of macromolecular conjugates have been developed and tested [2]. Much interest has focused on antibodies to the transferrin receptor [53]. This receptor is present on most proliferating cells but is expressed in high numbers on tumour cells [55]. Studies *in vitro* using the monoclonal antibody B3/25 attached to HPMA demonstrated one of the main difficulties in using antibodies. The conjugate was no more actively internalised than one containing non-specific IgG, showing that conjugation had eliminated the specificity of the antibody [52]

Other workers [56,57,5859,60] have looked at targeting of co-polymers bearing anti-Thy 1.2 antibodies which interact with Thy 1.2 alloantigens on mouse splenic cells. From these studies it became apparent that the binding method used was of great importance in retaining activity [60]. The targeting of HPMA co-polymers to colorectal cancer has also been investigated using Fab' fragments of specific antibodies [61].

An alternative approach was investigated by Shen *et al* [62]. They developed a conjugate of poly-D-lysine where the antibody was bound *via* the Fab' regions to expose only the Fc region. As many cells exhibit Fc receptors, the polymer can act as a hapten for interactions. Many tumour cells show Fc receptors including a few which are derived from cells which do not have this receptor type. This last type are of particular interest in drug targeting. The polymer was taken up more effectively than with non targeted polymers in Fc-positive cells but, as expected, showed no enhancement in Fc-negative cells.

1.3.4.3.3 Hormonal targets

A further consideration is the use of hormone receptor interactions to promote internalisation and drug targeting. The melanocyte stimulating hormone receptor (α-MSH receptor) is of interest, as this receptor is expressed by the majority of

melanocytes in malignant melanoma [63]. Malignant melanoma is a cancer which is difficult to treat, often leading to hepatic and brain metastases [63]. O'Hare *et al* [63,64,65] have used HPMA co-polymers bearing doxorubicin and MSH to asses hormonal targeting. Effective targeting of the polymer was demonstrated both *in vitro*, in B16F10 cells, a cell line which exhibits α-MSH receptors, and *in vivo*, in mice inoculated with B16F10. Binding of the hormone, to the polymer, through its N-terminus, did not affect activity. This is not surprising as the receptor binding site of this 13 amino acid peptide is the region between the sixth and tenth residues which are unaffected by conjugation to the polymer. Although these results are promising, workers have recently shown that that receptors for MSH are expressed in other cell lines, particularly in the brain, which may limit the therapeutic opportunities for these conjugates [66,67].

## 1.4 Lysosomal Degradation

Once a drug molecule is only able to pass into a cell by endocytosis, as in the case of a macromolecular pro-drug, the final destination of that macromolecule is pre-determined to be the lysosome.

Lysosomes are the organelles of cells responsible for digestion and are found in almost all mammalian cells [28]. The lysosomal membrane is impermeable to macromolecules and most initial products of digestion such as oligosaccharides. However, it is freely permeable to single units such as amino acids, monosaccharides and mononucleotides [28]. The lysosomal membrane also contains specific porters for some amino acids to allow rapid transport of the degradation products to the cytoplasm [68].

Lysosomes exhibit unique characteristics in terms of pH and enzyme activity. Lysosomal pH is slightly acidic, usually about pH 5.5, in comparison to serum [34]. This is an important consideration as the drug delivered should not be acid sensitive. Lysosomes contain over 50 digestive enzymes, the majority of which are hydrolytic in nature. They allow the cleavage of all common natural inter-monomer bonds such as

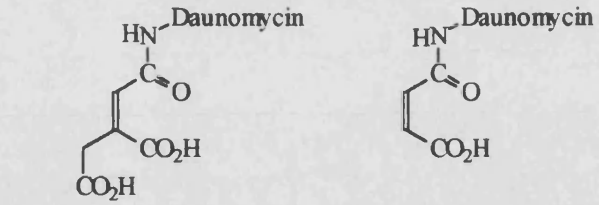
peptidyl, glycosyl and phosphate linkages [54]. One family of enzymes found in lysosomes are of particular interest, the cathepsins. The thiol-proteinases, cathepsin B, H, and L are involved in peptide cleavage as are the non-thiol dependent cathepsin C, a peptidyldipeptidase, and cathepsin D, an endopeptidase [15,69].

These characteristics of lysosomes afford the means of selective release of active drug from its macromolecular carrier within the cell, conferring the highest level of targeting of drugs. Release of the drug can be achieved by exploiting the acidic pH of lysosomes, the specific and high level enzyme activity or the reductive properties of the endosomal pathway [3].

Many workers have shown that release of a drug from a macromolecule is better achieved by incorporating a spacer unit between the macromolecule and drug [16]. Otherwise, the macromolecule itself must be degradable, a possibility which will be discussed in Chapter 2. The ideal properties of the linkage are that it should render the drug inactive, that it should be stable in serum and be readily broken down in lysosomes. Both covalent and non-covalent linkages are acceptable. However, a non-covalent linkage, although easier to form, is unlikely to be as stable in serum as is a covalent linkage [10].

### 1.4.1 pH sensitive Linkages

Shen [70] first explored the potential of acid labile spacers in 1981 using a cisaconityl acid spacer between daunomycin and poly(D-lysine) . Conjugates bearing this linkage demonstrated 90% growth inhibition in WEHI-5 cells, whereas polymers with N-maleyl spacers had no effect [70] (Figure 1.5).

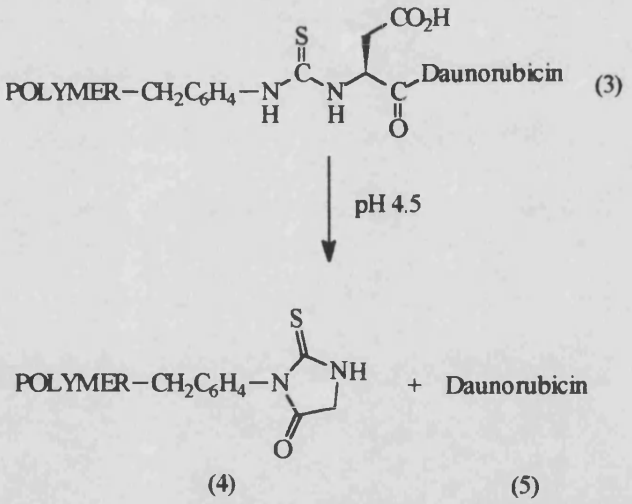



N-*cis*-aconityl daunomycin (1)

N-maleyl daunomycin (2)

Figure 1.5

A further example of acid labile spacers are those based on p-benzylthiocarbamoyl moiety (Scheme 1.1) [71]. In slightly acidic conditions, as in a lysosome, this spacer undergoes an Edman cyclisation, resulting in release of daunomycin (Scheme 1.1). This is only achieved if the amino acid used is acidic, such as aspartic acid. Studies comparing the side arm attached to aspartic acid or leucine demonstrated the suitability of the linkage. With aspartic acid, no drug release was seen in serum over the three weeks of the study, but at pH 5 and pH 4 release was afforded. The leucine derivative did not undergo cyclisation to form a thiazolinone at pH 7.4 or pH 5 and only a small amount of release was seen at pH 4 [71].




Scheme 1.1

More recently, Shen [72] has suggested that acid-labile drug arms are relatively unsuitable as the difference in pH between serum and lysosomes is not large enough

to provide totally site-specific degradation. In a novel approach, he has investigated the potential of non-covalent acid-sensitive linkages. The receptor-substrate complexes on the surface of cells are robust but are easily broken down in lysosomes. This is due to a conformational change in the receptor at the lower pH resulting in a decreased binding affinity for the substrate. Shen has developed a polymer poly (glutaminylhistamine-glutamate) (PHG) which forms a pH-dependent complex with poly(lysine). Release of poly(lysine)-methotrexate from PHG was demonstrated in lysosomes [72]. Obviously, further work is required on this approach before it can be utilised.

### 1.4.2 Disulphide linkages

The degradation of proteins is often achieved by the reductive cleavage of disulphide bonds and it is thought that this reaction is the first step in the degradation of insulin. The actual location of this reaction is unknown, but it is believed that it occurs early on in the endosomal pathway, as shown by studies on diphtheria toxin. Diphtheria toxin consists of two sub-units A and B linked *via* a disulphide bond. Release of the active B portion is afforded by cleavage of the bond, but as this portion of the toxin is acid-sensitive, this cleavage and release must occur before reaching the lysosome [16,62].

Shen *et al* [16,62] have investigated disulphide bonds for the release of methotrexate from poly-D-Lysine. Poly-D-lysine is an ideal choice of polymer as it is not degraded by lysosomes and any drug release must result from cleavage of the disulphide bond. The conjugate was effective in both normal and methotrexate-deficient cells and was unaffected by leupeptin or decreased glutathione levels; however, its activity was abolished by 2-mercaptoethanol. These results demonstrate that cleavage does not occur on the cell surface, is independent of enzymes and acidic pH and is not a non-enzymatic glutathione reaction. This confirms the view that the reduction occurs in pinosomes prior to fusion with the pre-lysosome.

More recently, Bonfils *et al* [73] have used disulphide bonds to link oligonucleotides to mannosylated proteins. These conjugates were stable in incubation with serum but rapidly released the nucleotide within cells.

### 1.4.3 Lysosomal Enzymes

Most interest in the development of spacers has been focused on amino acid sequences which are specific to lysosomal degradation by enzymes. One of the earliest examples of this approach dates from 1954. Jatzkewitz employed a dipeptide sequence GlyLeu to attach mescaline to poly(vinylpyrrolidone-co-acrylic acid). This increased the biological half life of mescaline from a few hours to seventeen days [54,74].

The specificity of lysosomal enzymes can be described using the method of Schechter and Berger [75]. The substrate specificity of many enzymes is now known enabling the design of spacers for testing. Two major research groups have investigated the action of lysosomal enzymes in the context of macromolecular pro-drugs : Duncan and Kopecek *et al* and Trouet *et al.*

#### 1.4.3.1 Work of Duncan and Kopecek

##### 1.4.3.1.1 Model studies on chymotrypsin

Initially macromolecular pro-drugs based on HPMA were synthesised to contain peptide sequences with known specificity for chymotrypsin to investigate the effect of the macromolecule, the length of spacer, and the stereospecificity of the amino acids of the spacer on enzyme specificity. The suitability of the sequence was measured by the release of a drug model - 4-nitroaniline [75].

The binding site of chymotrypsin displays a preference for an amino acid which can act as a hydrogen bond donator in the $P_1$ position, one able to form van der Waal's interactions with $Ile^{99}$ at $P_2$ and hydrophobic amino acids at $P_4$ [75]. These specificities were retained, where the peptide bond to be cleaved was between a macromolecule

and a drug molecule, revealing major differences in the release of drug from spacers of the same length [75].

With smaller spacers, for example those with only two amino acids, substrate specificity was of little importance. In this case, the polymer backbone will occupy some of the binding site, so decreasing the strength of binding. The degradation was also affected, not surprisingly, by the inclusion of unnatural D amino acids such as D-Phe and D-Ala. In these cases degradation was greatly decreased [75,76].

The effect of the macromolecule on enzyme action has been further investigated using PVP and PEG [77,78]. Conjugates were synthesised with the same peptide sequences as previously produced for HPMA and the degradation rates were measured. The same relative degradability of the sequences was seen in all three series; however the rates of degradation of a sequence varied considerably. The highest rates of release were seen in the PEG series in that even a one amino acid spacer was degradable. It is thought that this may be due to enhanced interactions between PEG and the enzyme. PEG is a linear polymer with the ability to form hydrogen bonds. Ulbrich *et al* [78] have proposed possible interactions between PEG and the enzyme binding site (Figure 1.6).

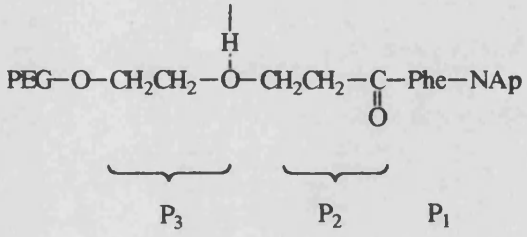



Figure 1.6

A higher rate of degradation was also evident with PVP, although this was not as impressive as that of PEG [77]. This is likely to be due to an interaction of the cationic chymotrypsin with the negatively charged polymer. These studies also provided evidence that increased levels of substitution lead to decreases in degradability. This is likely to be caused by decreased enzyme accessibility and suggests that there may be an optimum level of side chain incorporation [77].

1.4.3.1.2 Experiments using cathepsins

Based on this early work, attempts were made to design peptide linkages which are substrates for particular lysosomal cathepsins [15]. Studies were performed using a wide variety of single enzymes and mixed lysosomal extracts [15,54,48,69,79] but the most promising results were achieved in studies on cathepsin D. Cathepsin D is an endopeptidase with specificity for the bond between hydrophobic amino acids [15]. On the whole it has little activity with low molecular weight substrates, however, GlyPheLeuGlyPhe is a known substrate. Three sequences were prepared, GlyPheLeuGlyPhe, GlyGlyPheLeuGlyPhe and GlyPheLeuGly, and all degraded easily in a mixture of lysosomal enzymes. The first two sequences yielded Phe-NAp. This is not ideal, since release of unmodified drug would ensure target activity whereas amino acid conjugates may prove to be inactive. In the final sequence, however, linear release of unmodified drug was achieved. Interestingly, although this sequence was designed for degradation by cathepsin D it also fits the substrate conditions for cathepsin L activity *i.e.* hydrophobic amino acids in positions $P_2$ and $P_3$ and is degraded on incubation with cathepsin B [69,80]. Analysis of the degradation events showed that the terminal NAp bond was cleaved first to reveal a tetrapeptide which was further broken down to release LeuGly; the final amino acids GlyPhe were not cleaved from the polymer [15].

These studies [69] provided GlyPheLeuGly as a front line candidate for a lysosomally degradable sequence. The stability of HPMA co-polymers bearing this sequence in serum was determined. Serum contains a wide range of enzymes including a small amount of lysosomal enzymes which have leaked from cells, although most of the enzymes have trypsin-like activity *i.e.* they cleave bonds following Arg and Lys. The co-polymer was not degraded in serum and so was further evaluated [80].

Macromolecular pro-drugs were then prepared incorporating this sequence or the sequence GlyGly which is not degraded by lysosomal enzymes. Numerous drugs including daunomycin, adriamycin [81,82], sarcolysin [48], melphalan [83] and

doxorubicin [63,84,85], have been attached to this sequence and in all cases the degradability of GlyPheLeuGly is maintained in lysosomes whilst being minimal in serum. It has also been shown, by the activity of the conjugates, to be degraded in the lysosomes of many cell lines including B16F10-melanoma cells [63,85], L1210-mouse leukaemia cells [46.49,50,81], Walker's sarcoma cells [83,84] and the $HepG_2$-hepatoma cell line [31].

However, there are some problems which occur with this sequence. Firstly, it is slightly hydrophobic, and in sufficient quantities, could alter the nature of the macromolecule. This would be especially problematic if a hydrophobic drug were then to be attached [86]. Also, in some systems, release of free drug molecules is not achieved, only amino acid derivatives being released. This is probably due to the macromolecule used *e.g.* PHEG [87] and its interactions with the enzyme. Nevertheless as has been mentioned earlier, amino acid pro-drugs could be exported from the lysosome and result in lack of activity [88].

1.4.4 Work of Trouet *et al.*

Originally Trouet investigated macromolecular drug delivery using DNA as a carrier molecule. DNA is biodegradable so no spacer moiety was required to afford drug release [89]. However, the limitations of DNA as a carrier (Chapter 2) led to the use of albumin [90]. Release of the drug from albumin was not achieved from a direct linkage therefore spacer units were investigated. Following experiments with small amino acid pro-drugs, the ideal bond for release of daunomycin was found to be one between leucine and the drug and the ideal sequence for enzyme specificity to be AlaLeu [91,92]. Pro-drugs were then synthesised with one of three linkages AlaLeu, LeuAlaLeu, and AlaLeuAlaLeu [90]. Studies *in vitro* showed release to be highest from the tri-and tetrapeptide sequences and these conjugates were active in studies *in vivo*. The conjugates were stable in serum for 24 hours and this, in combination with the high lysosomal degradability, has lead to this sequence being used in a variety of situations [90].

De Mare *et al* [87] have developed spacer sequences based on AlaLeu to attach mitomycin C (MMC) to PHEG. Two sequences were sensitive to lysosomal hydrolysis GlyPheAlaLeu and AlaLeuAlaLeu resulting in release of MMC although some LeuMMC was also released. The sequence GlyGlyGlyLeu has also been used as a linker between PGA and adriamycin but, again, some amino acid derivatives are produced suggesting that the sequence has low substrate specificity [86].

The results from both research groups suggest that enzymatically degradable spacers provide the greatest selectivity in degradation and hence the greatest potential for targeted drug delivery. At this time, one macromolecular pro-drug incorporating the amino acid sequence GlyPheLeuGly is undergoing Phase II clinical trials in humans.

## 1.5 Conclusion

Macromolecules accumulate in many solid tumours due to a number of inherent physical properties of the tissue. This accumulation can be exploited to allow enhanced delivery and targeting of macromolecular pro-drugs to solid tumours. Altering the charge or size of the macromolecule can lead to enhanced uptake into target cells, as can the incorporation of targeting moieties. Lysosome specific delivery can be achieved by preparing a conjugate which affords release of a drug, by breakdown of the spacer, only within the lysosomal compartment.

# CHAPTER TWO

## MACROMOLECULAR PRO-DRUGS

### 2.1 Introduction

When considering a macromolecular pro-drug, the major determinant of *in vivo* characteristics is the macromolecule rather than the drug or targeting moiety. The ideal characteristics of a macromolecule are [11,93]:

1. Ease of synthesis
2. Ease of chemical modification
3. Water solubility
4. Biodegradability
5. Lack of toxicity
6. Lack of immunogenicity

However, at present, no carrier has been found which has all these characteristics. In this chapter, the most well known polymers are evaluated with respect to these criteria and previous macromolecular drug conjugates are discussed.

Both synthetic and natural macromolecules have been used as carriers. Synthetic polymers are considerably more versatile than non-synthetic polymers. They can be easily synthesised in known molecular weight fractions and modified for the attachment of drug molecules. They are also less toxic and immunogenic than natural polymers. Unfortunately, synthetic polymers are, on the whole, not degradable due to their carbon-carbon backbone. Thus, investigators are presently developing co-polymers of synthetic polymers and natural amino acids, which retain the favourable properties of synthetic polymers whilst allowing a limited biodegradability [11,93].

## 2.2 DNA

DNA was one of the first macromolecular carriers investigated. It is an ideal lysomotrophic carrier as it is stable in serum but is degraded in the lysosome. In the early 1970s, Trouet *et al.* [10] produced complexes of daunomycin and adriamycin intercalated into DNA. These complexes proved effective in *in vitro* studies in leukaemia cells and in $DBA_2$ mice inoculated with the L1210 leukaemia. Limited clinical trials were performed in terminal leukaemia patients whose disease was resistant to all forms of therapy. The complex was well tolerated and gave very encouraging results; in 20 cases of non-lymphoblastic leukaemia, 12 resulted in complete remission. Interest in DNA as a carrier has waned since then, possibly because of the instructional nature of DNA which may lead to unwanted side effects, or due to the difficulty of preparing drug conjugates [10,89].

## 2.3 Albumin

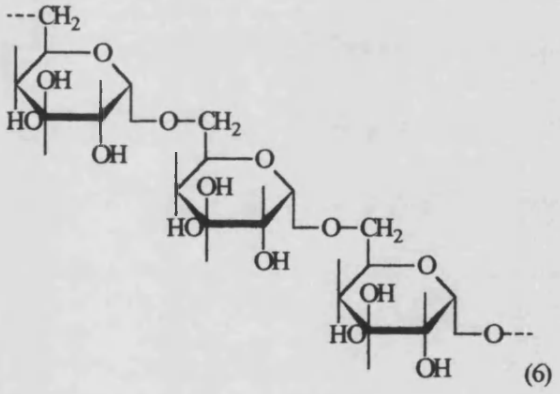
The serum protein, albumin, has also been used as a carrier. It is attractive due to good biological stability, lysosomal degradability, ease of chemical substitution, low toxicity and with homologous albumin, low immunogenicity. Early attempts to produce a methotrexate derivative of albumin resulted in an inactive conjugate [11]. Later attempts to produce a conjugate of daunomycin, attached via degradable spacers to albumin, resulted in therapeutic activity. The conjugates led to a 200% increase in life span in mice inoculated with L1210 [11,20,90].

## 2.4 Polysaccharides

Many polysaccharides have been used as macromolecular carriers including cellulose and starch [12]. However, dextran and inulin have been most widely investigated. Polysaccharides are attractive as carriers, because they are easily chemically derivatised and are of both low toxicity and immunogenicity.

### 2.4.1 Dextran

Dextran **(6)** is the collective name for a wide range of polysaccharides based on α-D-glucose having a predominance of 1,6-α linkages *i.e.* there is little chain branching (Figure 2.1). These polysaccharides are derived from bacteria and the actual composition is determined by their origin. One type is of particular interest in pharmaceutical applications and is derived from *Leuconostoc mesesteroides*. In this type, 95% of the chain is unbranched and the remaining 5% is branched with the majority of the branches only one or two residues long [12].




(6)

Figure 2.1 : Dextran

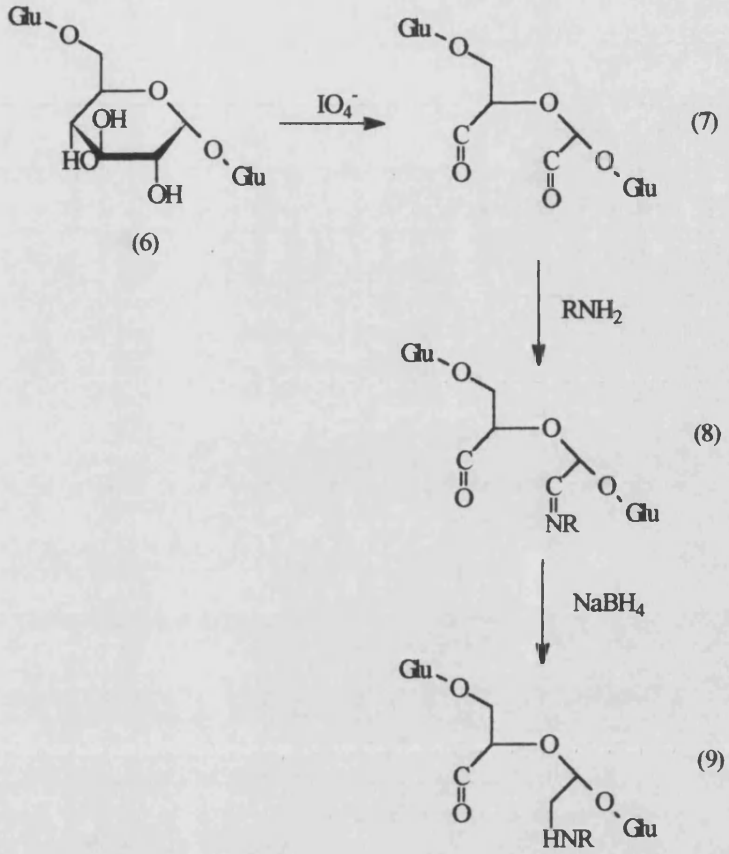
Dextran has been used for many years in medical practice. Dextran T-70 (dextran of molecular weight 70,000) is used as a plasma expander in patients suffering from shock or haemorrhage and dextran T-40 is used to increase blood flow in capillaries in the treatment of vascular occlusion [12]. Thus, dextran is an ideal candidate for a macromolecular carrier as its toxic and immunological effects are well characterised.

Dextrans are available in narrow molecular weight bands with low polydispersity that allow easy characterisation and evaluation of resulting drug conjugates. They also have very high water solubility, which is retained even with 20% w/w ligand incorporation [94].

2.4.1.1 Activation

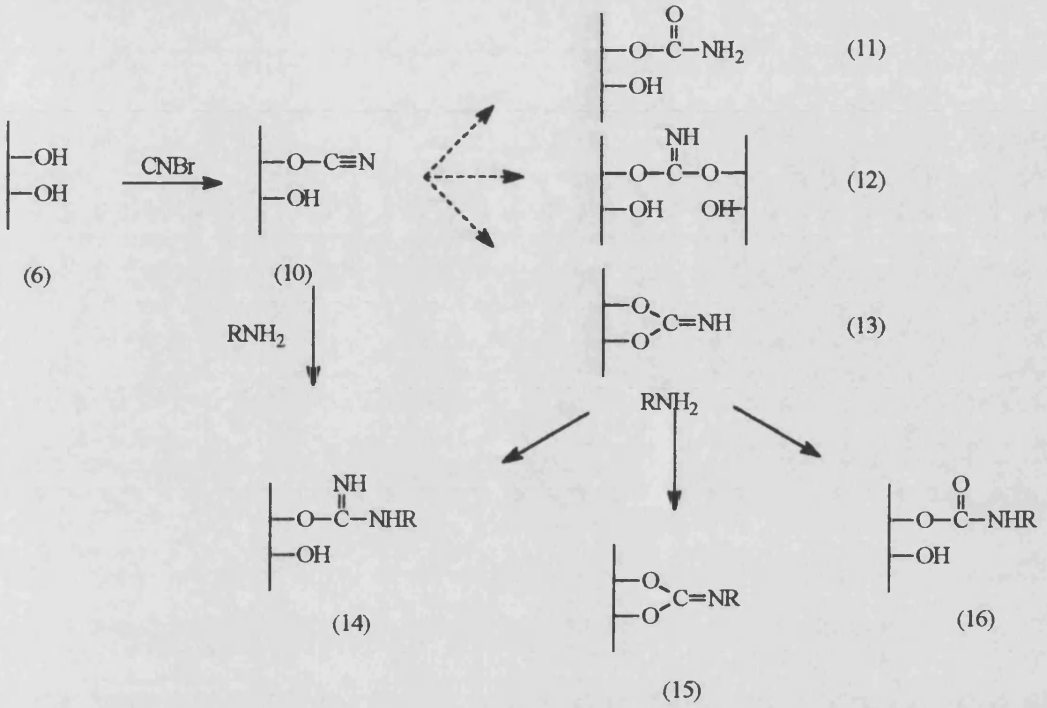
The methods for activation of dextran have been reviewed excellently by Larsen [12] and by Schacht [94]. Dextran contains a large number of hydroxyl groups that are suitable for either direct conjugation with carboxylic-acid bearing drugs, such as aspirin or for chemical modification and activation prior to coupling with other drugs. There are three main methods of activation, all of which have been used in the preparation of macromolecular pro-drugs.

The most popular method involves the production of dextran dialdehydes by the action of periodic acid (Scheme 2.1). The resulting aldehydes can then react to form Schiff bases with drugs containing amine groups. This bond is highly unstable and can be stabilised by reduction with sodium borohydride. This coupling and subsequent reduction can be achieved in one step. Unfortunately, this reaction may also result in the formation of hemiacetals that reduce the degree of oxidation along the chain and potentially affect coupling reactions. Chemical modification of this type also greatly effects the structure of the carrier backbone which will effect its physiochemical properties and potentially its toxicity and immunogenicity [12,95].



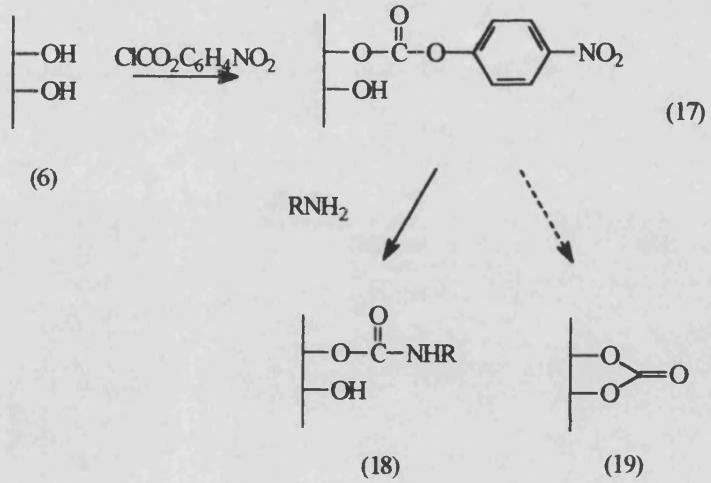

Scheme 2.1

Another attractive method is the use of cyanogen bromide. This leads to less alteration in the basic structure of the carrier, but does result in the introduction of positive charges into the molecule (Scheme 2.2) [12]. The reaction is not well understood but it is thought that, along with the desired electrophile, three other preliminary products are produced. The imidocarbonate structures are commonly produced in dextran activation, particularly the stable five-membered ring structure **(13)**. This can then form the desired linkage when treated with an amine but can also form other linkages which can result in a charged dextran. Thus, this approach, although the chemistry is easy to perform, is of limited use as reliable and reproducible ligand-carrier structures are not formed [12,94].




Scheme 2.2

The final method of activation is through the use of carbamate esters. Treatment of dextran with 4-nitrophenyl chloroformate results in the highly active carbonate which couples easily with amine drugs [95]. Again it is thought that some inter-chain cross-links are formed due to the close vicinity of the hydroxyl groups. (Scheme 2.3)

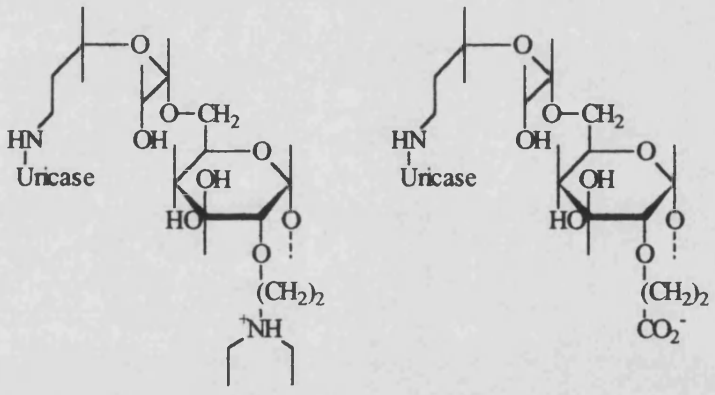



Scheme 2.3

2.4.1.2 Distribution *in vivo*

Dextran has a renal threshold of approximately 55,000 and fractions of a higher molecular weight undergo slow partial depolymerisation due to the action of dextranases [12]. Studies with dextran T-40 demonstrate a rapid clearance from the bloodstream; this is thought to be due to three excretory mechanisms. The main fraction which is below the glomerular filtration threshold is excreted in the urine. A small amount passes into the gastro-intestinal tract where it is degraded in the colon. Another small fraction passes across the endothelium and into the interstitium where it is recycled *via* the lymphatics. The remaining fractions are taken up over time into cells of the reticulo-endothelial system, such as macrophages and Kupffer cells of the liver, where the polysaccharide is slowly degraded [12].

The distribution of dextran *in vivo* can be greatly changed by chemical modification. A cationic derivative, diethylaminoethyl dextran, can be formed by treatment of dextran with diethylaminoethyl chloride and an anionic form, carboxymethyl dextran, by treatment with chloroacetic acid (Figure 2.2) [93,96].




Diethylaminoethyl dextran (20)

Carboxymethyl dextran (21)

Figure 2.2

Equally, incorporation of a spacer unit for attachment of a drug molecule can result in the formation of cationic and anionic derivatives of dextran [93]. The conjugation of mitomycin C (MMC) to cyanogen bromide-activated dextran *via* a 6-aminohexanoic

acid spacer results in a positively charged dextran molecule, whereas 6-oxyhexanoic acid spacers give a negatively charged polymer [12,93,97] (Figure 2.3).

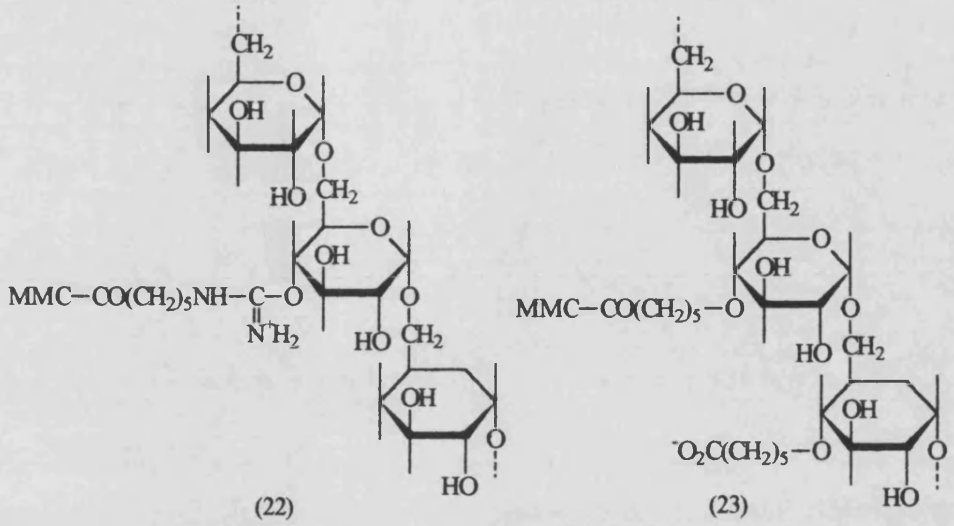



Figure 2.3

Studies on both these types of charged polymers reveal altered *in vivo* distributions. A cationic conjugate with uricase was rapidly cleared from plasma whereas an anionic derivative has an increased half-life. Measurements of hepatic uptake and urinary excretion gave similar results. Uricase was taken up faster when conjugated to neutral dextran. This rate was enhanced by conjugation to cationic dextran and reduced by conjugation to anionic dextran [96].

With the mitomycin C derivatives [97], studies were undertaken in tumour-bearing mice. Here the cationic derivative was rapidly cleared whereas the anionic derivative had an increased blood residence time, resulting in accumulation in the tumour. These results suggest that intentional incorporation of anionic charges into a dextran macromolecular pro-drug would result in an enhanced delivery system.

An alternative approach to the modification of distribution *in vivo* is the use of lectins to target a polymer to particular cell lines. In early studies, Vansteenkiste [44] investigated the effect of pendant D-galactosylated dextrans on distribution. Plasma clearance was higher than for non-derivatised dextran and hepatic uptake was

increased. An interesting finding from this study was that dextrans carrying fluorescein thiocarbamate alone also exhibited an increased disposition in liver. Thus, it can be taken that the changed *in vivo* distribution due to changes in the carrier could be augmented or overridden by attachment of drug.

2.4.1.3 Biodegredation

Dextran is degraded *in vivo* by the action of dextranases. These enzymes are absent from blood but are present in the liver, kidney, spleen and parts of the gastro-intestinal tract. Dextranases can be either *exo* or *endo* in their action. Dextran is only degraded very slowly by these enzymes and large molecular weight dextrans can be captured by the reticulo-endothelial system before they are degraded. Thus, it is important to consider degradation by isolated dextranases and tritosomes of both dextran and dextran derivatives [12].

In initial studies, Schacht *et al.* [95] investigated the degradation of dextran derivatives *in vitro* by an *endo* dextranase. Three derivatives were assessed, a reduced dialdehyde dextran, formed from periodate activation and reduction, an amine derivative, formed by the reaction of 2-hydroxypropylamine with chloroformate-activated dextran, and a succinate derivative. Degradation was diminished by periodate activation and this reduction was proportional to the degree of activation. The other derivatives gave similar results suggesting that enzyme activity is affected by the degree of derivatisation, rather than the type of modification. The degradation of these macromolecules in tritosomes was also assessed. Dextran itself was slowly degraded and both the urethane and succinylated dextrans showed even lower degradability. Interestingly, the reduced dialdehyde had, in this case, a higher rate of degradation. This was thought to be a combined effect of both hydrolytic and lysosomal degradation. These workers suggested that the degradation of dextran in lysosomes is achieved by dextranases with both *endo* and *exo* characteristics [95].

The same workers have also investigated the lysosomal degradation of dextran, cationic dextran dialdehyde and anionic dextran monosuccinate ester [98]. Results were

obtained for both the degradation index and degree of glucose release, *i.e.* the extent of *exo*-dextranase activity. Liberation of glucose was the same for both dextran and the derivatives. However, the degradation rates of the derivatives were lower. This confirms that dextran is degraded in lysosomes by more than one enzyme. It was proposed that the enzyme with *exo* activity was α-glucosidase and the *endo* enzyme was 1,6-α-D-glucose-6-glucanohydrolase.

Crepon *et al.* [99] have examined the degradation of dextran derivatives that bear more resemblance to macromolecular pro-drugs. Carboxylic acid, benzylamide, and sulphonated benzylamine derivatives of dextran T-40 were prepared and degraded using an *endo*-dextranase (Figure 2.4). Incorporation of increasing numbers of carboxylic acid groups resulted in a linear reduction in degradation. Comparison of polymers derivatised with carboxylic acid groups and polymers derivatised with both carboxylic acids and benzylamine sulphonated groups showed the benzylamine sulphonated groups had a greater inhibitory effect on lability to dextranase. Interestingly, some dextranase action still occurred even at 80% substitution [99].

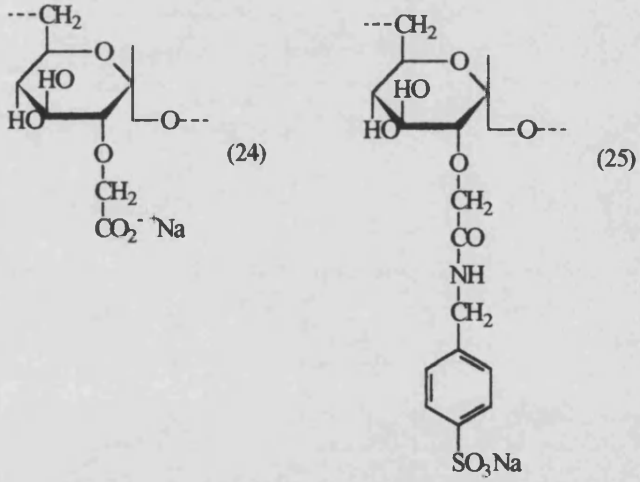



Figure 2.4

The effect of larger substituents has also been investigated [100]. Dextrans have been prepared bearing pendant poly(ethylene glycol) chains which were degraded both with dextranase and tritosomes. All the derivatives were degraded by dextranase but the extent of degradation was substantially reduced with longer poly(ethylene glycol)

chains. With the lysosomal enzymes, no degradation of the derivatives was seen in 36 hours.

These results suggest that dextran is suitable as a carrier molecule, as it is not degraded in the blood stream where there are no dextranases, but is very slowly degraded in lysosomes. To afford efficient release of drug from dextran, it may be appropriate to use lysosomally degradable spacers.

2.4.1.4 Immunogenicity

The immunogenicity of dextran has been reviewed by Rihova and Riha [101]. Immunogenicity is dependent on the molecular weight of the dextran. Dextrans with MW 51,000 Da have very low immunogenicity whereas injection of dextran with MW over 90,000 Da results in the formation of antibodies over three weeks. It has also been shown that the immune effect depends on the dose injected, for large molecular weight dextrans, an immune response is induced at low doses but, at higher doses, tolerance is seen. The immunogenicity of dextrans is also structure-dependent, branched polysaccharides being more immunogenic [94].

Two main specificities of antibody are raised to dextran, one with a combining site for isomaltotriose and one with a larger combining site for isomaltohexose [101].

The immunogenicity of modified dextrans must also be evaluated, as this provides information on the macromolecular pro-drug situation. Crepon [99] evaluated the antibody response to a dextran T-40 derivative with 54% carboxylic acid groups and 19.5% sulphonated benzylamine units. The polymer was injected three times over 14 days, with or without complete Freunds' adjuvant, into Balb/c mice. The antibody titre was low, even in mice treated with the adjuvant, and the conjugate can be considered to have low immunogenicity. Despite the low immunogenicity of dextran, occasional anaphylactic reactions are seen. However, the development of a pre-immunisation technique has reduced the risk to 1 in 85,000 [12].

2.4.1.5 Macromolecular pro-drugs based on dextran

Sezaki *et al.* have developed pro-drugs of mitomycin C based on dextran [102]. These workers employ hydrolysable spacer bonds, rather than lysosomally degradable bonds, in order to achieve release of the acid-unstable drug both within the cell and within the tumour mass. This could however result in the release of the drug in other regions of the body, with consequent toxicity.

Mitomycin C has been attached to cyanogen bromide-activated dextran thorough a variety of bonds [12,93,96]. In a recent study [102], three spacers of varying length were evaluated with respect to rate of drug release and effectiveness *in vivo*. *In vitro* drug release rates demonstrated an increased half-life with increasing chain length, but in cell culture experiments growth inhibition increased as the length of the spacer decreased. Thus these two effects must be balanced to provide the most selectively active conjugate. Studies *in vivo* have shown that a 6-aminohexanoic acid spacer provides the largest increase in life-span combined with the largest therapeutic index [102].

2.4.2 Inulin

Inulin is a vegetable-derived polysaccharide based on fructose units, which is terminated at one end with a sucrose unit. It has limited solubility and is degraded by acid. It is a small polysaccharide with MW < 10,000, and is used clinically for testing renal filtration rates as in a normal kidney as it is excreted rapidly without degradation [94].

Activation of inulin using the standard methods mentioned above proved unsuccessful. Periodate activation resulted in only 50% active units, due to the high level of formation of hemiacetals [94]. An alternative activation method has been developed which is also suitable for the activation of dextran [103]. Treatment with epichlorohydrin yields a 3-chloro-2-hydroxypropyl derivative which can react easily with amine drugs. Succinylated derivatives can also be prepared which can then be

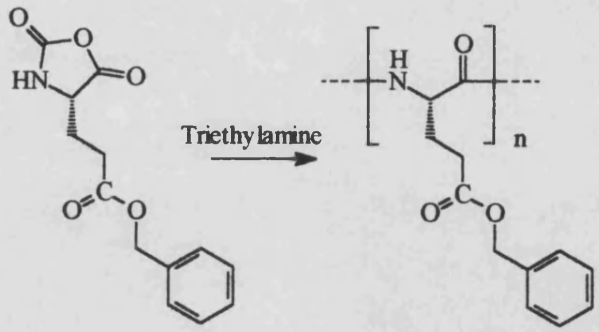
activated with 1,1'-carbonyldiimidazole [104,105]. Inulin has been used to a limited extent in preparing macromolecular pro-drugs, mainly as a carrier for the anti-arrhythmic drug, procainamide.[94]

## 2.5 Poly(amino acids)

Poly(amino acids) can be considered to be synthetic proteins. The use of these macromolecules as carriers has been explored for many years. The degradability of poly amino acids is a highly attractive feature as is the ease of chemical modification. However, the use of many macromolecules of this type is restricted due to their general cell toxicity. Polymers of most amino acids have been prepared and evaluated. The most popular systems are poly(glutamic acid), poly(lysine), poly(hydroxyethyl-glutamine) and poly(hydroxyethylaspartamide).

### 2.5.1 Poly(glutamic acid)

Poly(glutamic acid) (poly(Glu)) can be easily prepared by base-initiated polymerisation of the N-carboxyanhydrides of L-glutamic acid γ-benzyl ester (Scheme 2.4). Deprotection reveals the desired polymer. The polymerisation reaction can be controlled to produce polymers of a wide molecular weight range [106,107].

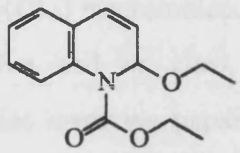



(26) (27)

Scheme 2.4

Although attachment of amine drugs to the carboxylic acid function would appear to be easily achievable using standard peptide chemistry, some workers have found

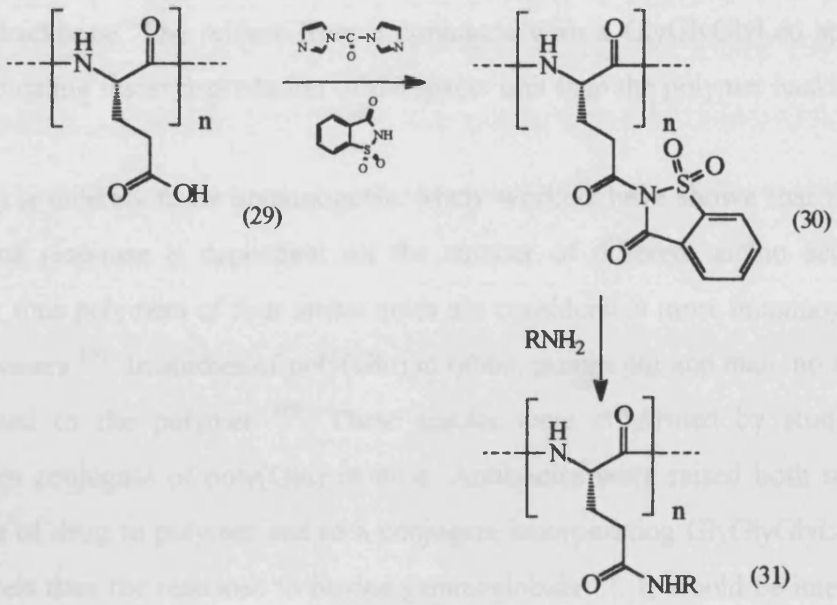
difficulties with activation procedures. Activation with carbodiimides can result in the formation of cyclic imides, which can lead to chain scission, or inactive N-acylisoureas. These problems can be overcome by using 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to form a mixed anhydride (Figure 2.5) [106,107].



EEDQ

Figure 2.5

The same workers have also developed an alternative coupling method for the attachment of peptide spacers to the macromolecule [86]. Poly(Glu) can be activated using a CDI-saccharin method and then coupled with tetramethylguanidinium salts of oligopeptides to produce the spacer [86,107] (Scheme 2.5). A stepwise incorporation of amino acids is also possible using a modification of this method, in which the diimidazole can be regenerated using phenyl dichlorophosphate. With both methods, the drug can then be coupled to the polymer *via* the EEDQ method [106,107].




Scheme 2.5

Complexes of poly(Glu) can also be prepared. The polymer has an inherent negative charge and a non-covalent complex of this and *cis*-dichlorodiammineplatinum II has been made and evaluated as a pro-drug [108]

Hoes *et al* have investigated poly(Glu) macromolecular pro-drugs of adriamycin with respect to their activity *in vivo* and *in vitro*, rate of release of drug and immunogenicity [86,106,107]. In studies involving papain *in vitro*, release of adriamycin was achieved only when a peptide spacer was incorporated between poly(Glu) and the drug molecule. This is to be expected, as papain has specificity for the cleavage of α-amide bonds. Further studies, evaluating the effect of spacer concentration gave surprising results. Cleavage of the spacer is expected to be faster in polymers with a few spacer units, as access of the enzyme is facilitated. However, with these conjugates, degradation proceeded faster with polymers bearing many chains. These authors considered these results to be due to degradation of the peptide backbone by the enzyme [109].

Later studies using lysosomal enzymes, tritosomes, confirmed many of these results. Release was obtained slowly in the direct conjugate, due to degradation of the polymer backbone. The release from a conjugate with a GlyGlyGlyLeu spacer was faster, indicating faster degradation of the spacer unit than the polymer backbone [86].

Poly(Glu) is unlikely to be immunogenic. Many workers have shown that the degree of immune response is dependent on the number of different amino acids in the molecule; thus polymers of four amino acids are considerably more immunogenic than homopolymers [101]. In studies of poly(Glu) in rabbit, guinea pig and man, no antibodies were raised to the polymer [109]. These results were confirmed by studies of an adriamycin conjugate of poly(Glu) in mice. Antibodies were raised both to a direct conjugate of drug to polymer and to a conjugate incorporating GlyGlyGlyLeu, but at lower levels than the response to bovine gammaglobulin [86]. It would be interesting to develop these studies further. Both adriamycin and spacer units have been shown previously to act as haptens. Thus the antibodies raised should be examined for cross-reactivity to determine whether their combining site is part of the polymer backbone.

The cytotoxicity *in vitro* and *in vivo* of these conjugates has also been evaluated [86,106]. Initial experiments, using both directly-bound and spacer-bound adriamycin, suggested that the compounds were completely devoid of activity against L1210 and B16 cell lines. However, carriers with a biodegradable spacer, when assessed *in vivo* in mice inoculated with B16 melanoma, gave increased survival times. Not surprisingly, the direct conjugate was also inactive *in vivo*.

### 2.5.2 Poly(Lysine)

Poly(L-lysine) would appear to be an ideal macromolecular carrier. It is available in a wide range of molecular weights, has a high number of amine groups for attachment of drug molecules, is biodegradable and can stimulate endocytosis due to the interaction of positive charges with the negatively charged cell membrane. Poly(D-lysine) has all these advantages except that it is not biodegradable, owing to its stereochemistry [110].

Poly(L-lysine) was used regularly in the early years of macromolecular pro-drug development. It was used mainly as a carrier for methotrexate and, in these studies, the intracellular mechanism of pro-drug action was confirmed, as conjugates were active in cells without transport mechanisms for methotrexate [16,62]. Poly(D-lysine) was also an excellent tool in the development of lysosomally degraded spacer arms. Release of a drug from a poly(D-Lysine) conjugate with a spacer can only occur if the spacer is degraded [16]. More recently, however, the use of both these carriers has declined due to their general cytotoxicity.

Poly(L-lysine) is known to have antibacterial, antiviral and antitumour activity. These properties are thought to be due to interactions with the cellular membrane or parasite coat. Unfortunately, the charged interaction that provides these positive effects can also lead to toxicity as it is not tumour-cell specific. In a comprehensive study, Choksakulnimitr *et al.* [111] investigated the effect of numerous macromolecules on cultured bovine brain endothelial cells, a model of the blood-brain barrier, mouse

peritoneal macrophages, a model of Kupffer cells, and rat hepatocytes. Damage to the cell membrane was measured by the release of lactate dehydrogenase (LDH). With Poly(Lys) of high molecular weight (39,880 Da), severe leakage of LDH was observed in all the cell lines, while with a smaller polymer (8,000 Da) release was reduced, especially in macrophages. It has been suggested that the flexibility of poly(Lys) plays a major part in the release, as many interactions can be made on the cell surface leading to membrane disruption. These results are particularly worrying as polycations have been shown to accumulate in the liver *in vivo* and suggest that the toxicity of poly(Lys) may be too high for use in macromolecular pro-drugs.

The immunogenicity of poly(Lys) is more debatable. In some animals, antibodies can be raised to the homopolymer but the response in a particular species is unpredictable [101]. Furthermore, initial studies by Maurer suggested that the polymer was non-immunogenic in rabbits, however it has now been shown that at low levels immune responses do occur [112]. In a recent study, the immunogenicity of poly(D-Lys) has been investigated in rabbits [112]. Antibodies of both IgM and IgG were raised in response to an initial inoculation with the polymer. On a further administration, a booster effect was seen consisting only of IgG antibodies. The antibodies were isolated and were only reactive against poly(D-Lys). No activity was seen with monomers, suggesting that at least part of the antibody determinant is the peptide bond, or with poly(L-Lys) showing stereochemical specificity. These results suggest that both poly(L-Lys) and poly(D-Lys) are slightly immunogenic. This does not preclude their use in macromolecular pro-drugs but it should be noted that the immunogenicity is likely to be enhanced by inclusion of spacers or drug molecules.

The concerns about the toxicity of poly(Lys) have led to the development of new approaches to using the polymer as a drug carrier. Monsigny *et al* [3] have investigated a conjugate of poly (L-Lys) with MDP and sugar targeting moieties in which any free lysine ε-amines have been acylated with δ-gluconolactone. This considerably reduces the adverse properties of the polymer whilst retaining the useful properties. Thus, the polymer is still degradable but is electrically neutral and has a higher water solubility [113,114]. Immunogenicity studies have shown that antibodies are not raised to

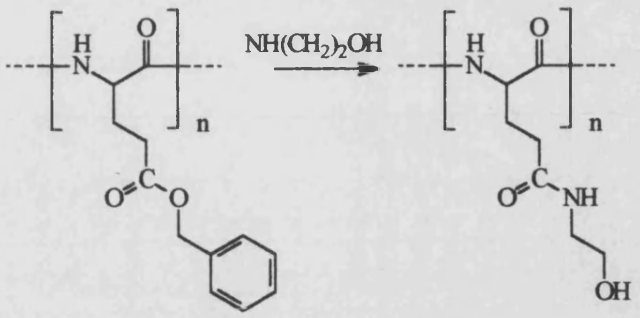
this conjugate [3] and that the neutralisation of charge results in a decrease of non-specific cell binding and toxicity.

Hudecz *et al.* have investigated branched polypeptides based on poly(Lys) as carriers [115]. Branched polypeptides have been used as synthetic antigens as the antigenicity of amino acid polymers is increased in polymers with many different components [101]. Thus, before branched polypeptides can be used as carriers, non-immunogenic structures must be found. In a large study of the toxicity of these carriers, it was found that inclusion of other amino acids onto a poly(L-Lys) backbone can reduce the toxicity to cell cultures, both rat liver and mouse spleen cells, whilst the positive cytotoxic effect against cervical cancer cells is maintained.

Hence, although poly(Lys) is not a suitable candidate for a drug carrier due to its high level of non-specific toxicity, modifications of the structure could lead to suitably non-toxic molecules.

### 2.5.3 Poly(hydroxyethylglutamate)

Poly(hydroxyethylglutamate) (PHEG) is a water soluble, neutral, biodegradable and biocompatible polymer that has been proposed as a plasma expander. It is easily prepared and can be reliably activated, making it a suitable candidate as a drug carrier. PHEG **(32)** can be synthesised from poly(γ-benzylglutamate) *via* an aminolysis reaction with 2-aminoethanol (Scheme 2.6) [116]. Treatment with 2-aminoethanol alone can result in chain scission due to aminolysis reactions on the backbone amides. This can be avoided by the use of the catalyst, 2-hydroxypyridine. With a five-fold excess of the catalyst, complete conversion was achieved with minimal chain loss [117].




(27) (32)

Scheme 2.6

The single hydroxyl group can be easily activated by treatment with 4-nitrophenyl chloroformate. In contrast to dextran, the desired carbonate ester is not rearranged and inter and intra chain cross-links do not occur. A useful property of this reaction is that the degree of activation is proportional to the concentration of chloroformate and thus can be tailored for particular requirements [116]. Quantitative couplings with the amino groups of both targeting moieties, such as sugars, and drugs, such as melphalan, can be achieved within 24 hours [116].

PHEG is known to be biodegradable. However, in an extensive study, Pytela *et al.* [118] have investigated the role of particular enzymes in this degradation. PHEG was incubated with both thiol proteases, such as papain and cathepsin B which have a lysosomal type activity, and serine proteases, such as chymotrypsin and elastase which are found in blood. Interestingly, the homopolymer was resistant to degradation by the serine proteases and was degraded by the thiol proteases, suggesting a potential lysomotrophic carrier. Unfortunately, when the same workers investigated the effect of inclusion of a hydrophobic group on the side chain, they found that the conjugate was susceptible to serine proteases. This could mean that the selectivity of degradation could be abolished by attachment of a drug. Not surprisingly, the degradation of PHEG was reduced by incorporation of some of the D-amino acid and abolished in D-PHEG.

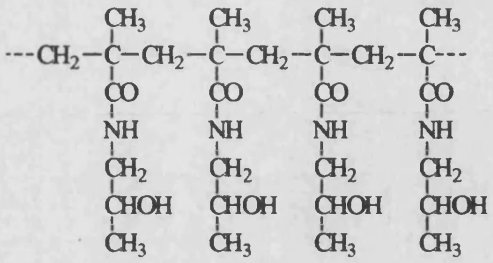
PHEG has been investigated mainly as a carrier of mitomycin C. Studies *in vitro* have demonstrated the potential of peptide spacers to afford release of the drug within lysosomes whilst retaining hydrolytic stability in the blood stream but the conjugates have not been investigated further [87]. Another approach in the use of PHEG is to co-polymerise it with other amino acids to produce macromolecules with different properties and biodistribution [118,119].

### 2.5.4 Poly(α,β-(N-(2-hydroxyethyl)-D,L-aspartamide)) (PHEA)

PHEA has also been evaluated as a plasma expander and a drug carrier, although it has not been widely used at present. PHEA has a similar chemical reactivity to PHEG, thus it can be activated to the nitrophenyl carbamate and coupled to amine-containing molecules such as sugars [51]. Coupling of carboxylic acid drug molecules can also be achieved by the use of reagents such as CDI [120]. For other drugs such as acyclovir, with a hydroxyl group, the drug molecule can be pre-activated by treatment with succinic anhydride. A CDI coupling can then be performed between the carboxylic acid group of the drug derivative and the hydroxyl of the polymer. Interestingly, this approach also results in the inclusion of a spacer unit [121].

## 2.6 Hydroxypropylmethacrylamide (HPMA)

Hydroxypropylmethacrylamide **(33)** and its co-polymers have been investigated as blood plasma expanders and drug carriers [122]. HPMA is a synthetic vinyl polymer produced by radical precipitation polymerisation (Figure 2.6) [123]. The homopolymer is non-immunogenic [101] and is easily activated to allow the attachment of drug moieties, making it an almost ideal macromolecule. One potential limitation of the system is its lack of biodegradability, which restricts the use to macromolecules with a molecular weight of less than the glomerular filtration threshold.

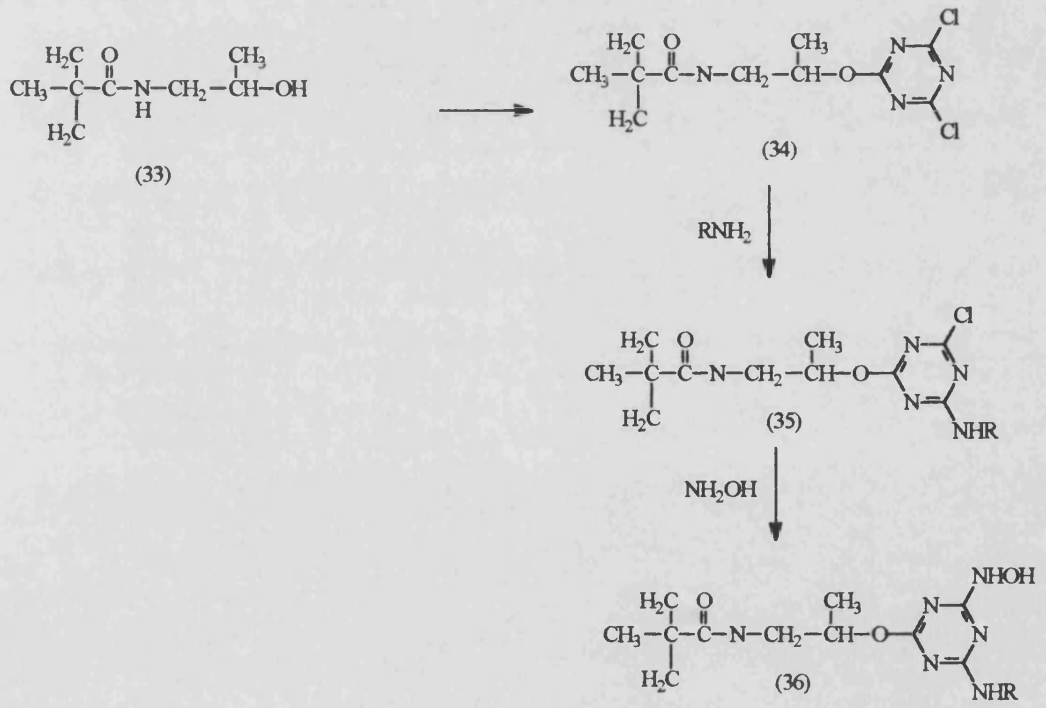


(33)

Figure 2.6 : HPMA

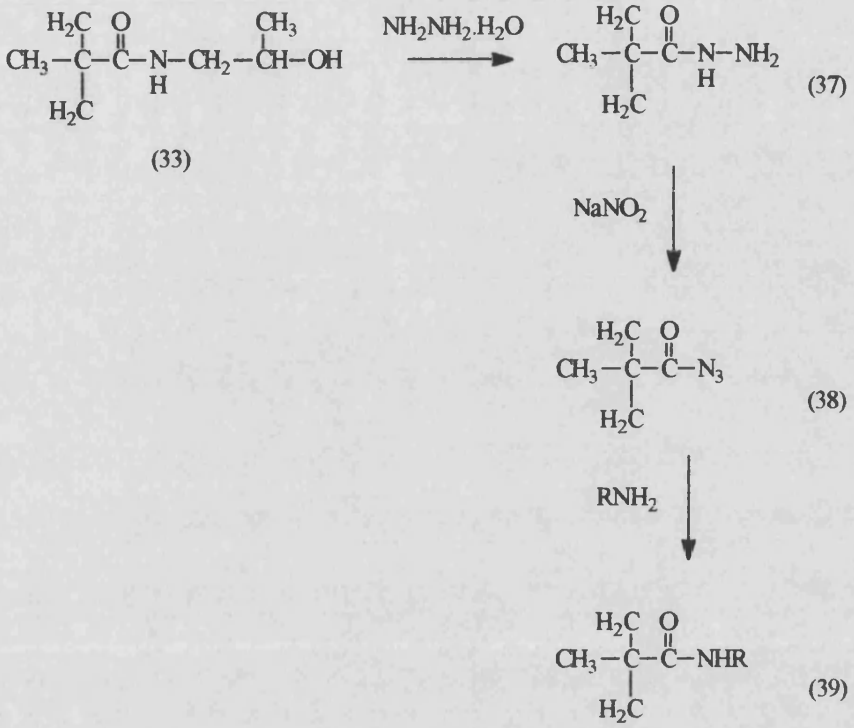
2.6.1 Activation

The homopolymer is itself inert. However, the hydroxyl groups can be activated to allow attachment of drugs and enzymes. One of the easiest methods of activation is to treat HPMA with cyanuric chloride (Scheme 2.7) [124]. The resulting active species reacts easily with enzymes in aqueous solution.



Scheme 2.7

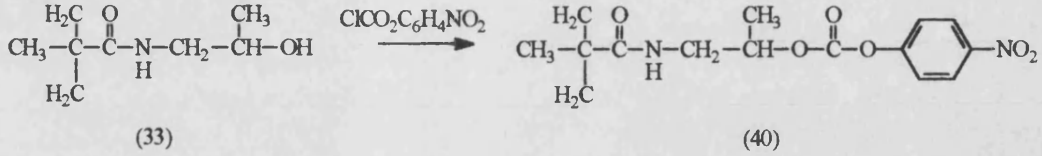
Hydrazide derivatives can also be prepared which, when activated to the azide, can be coupled to amine-containing drugs (Scheme 2.8) [124].




Scheme 2.8

Activation of the polymer can also be achieved by the use of cyanogen bromide [125]. Although this reaction is versatile and can be tailored for the degree of reactivity required, it can result in inter- and intra-chain reactions. All these methods do result in substantial changes in the structure and charge of the homopolymer that may affect both its distribution and immunogenicity *in vivo*.

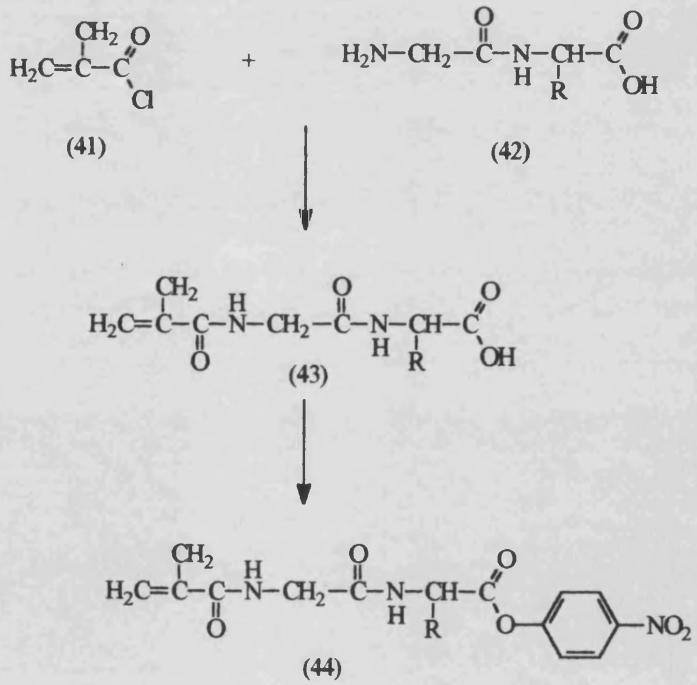
An alternative approach is to use an active ester method of activation [126]. Treatment of the homopolymer with 4-nitrophenyl chloroformate provides an active polymer. Aminolysis reactions with amino acids or drugs results in the restoration of the original polymer structure. This is the most useful and versatile method of activation available. (Scheme 2.9)



(33) (40)

Scheme 2.9

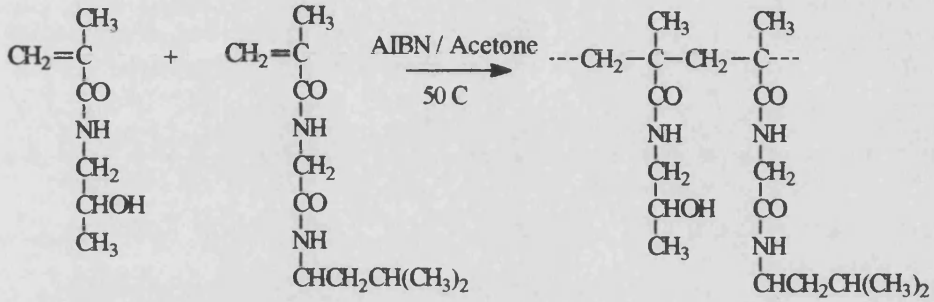
Activation of the homopolymer is, however, not suitable for reproducible levels of incorporation of drug. The alternative is to use a co-polymerisation method. Co-polymerisation of HPMA with derivatised methacrylamide units can be easily achieved. This allows a more accurate and reproducible interaction of reactive groups into the polymer whilst maintaining the majority of characteristics of the homopolymer. Kopecek *et al.* [123] have developed this approach and have used co-polymers of HPMA incorporating peptide spacers for the delivery of many drugs. The general method of synthesis involves the N-methacrylylation of an amino acid or dipeptide units under Schotten Baumann conditions. The α-amino acid can then be activated as a nitrophenyl ester (Scheme 2.10).



(41) (42) (43) (44)

Scheme 2.10

Co-polymerisation of these methacrylamide derivatives with HPMA occurs *via* a radical method involving an azo initiator such as α,α'-azobisisobutyronitrile (AIBN). The co-polymerisation takes place rapidly at 50°C in acetone. However, the rate of polymerisation and yield of polymer are reduced in comparison to preparation of the homopolymer [127]. Using this method, up to 10% reactive side chains have been incorporated [54] (Scheme 2.11).

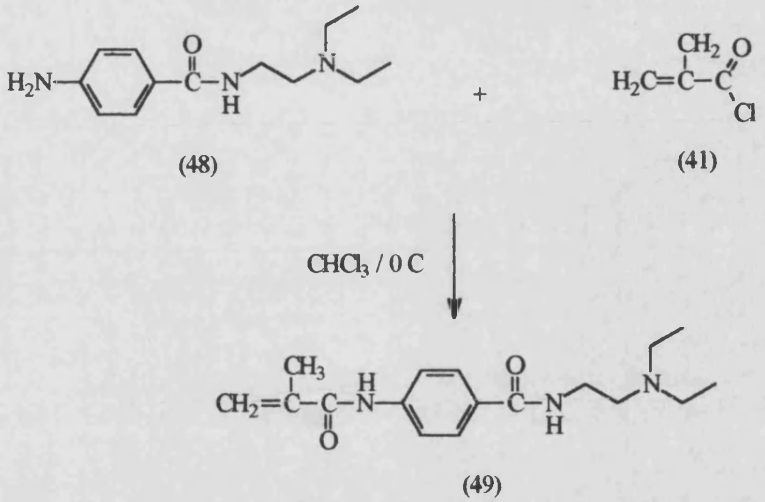



(45) (46) (47)

Scheme 2.11

Drug molecules or targeting moieties can be easily incorporated into the polymer by aminolysis reactions. The reactivity of the nitrophenyl groups on the polymer is considerably reduced in comparison to the monomers owing to steric effects [127]. The rate of reaction is also dependent on the nature of the ester, α-amino active esters react faster than ω-acylamino esters [128].

With some drugs, it is possible to produce a methacrylylated derivative that can then be co-polymerised. This approach has been utilised by Schacht for the anti-arrhythmic drug, procainamide. Co-polymerisation of the derivative with HPMA resulted in a water soluble drug conjugate (Scheme 2.12) [129]




Scheme 2.12

2.6.2. Distribution *in vivo*

Studies with iodine-labelled HPMA provide a value for the glomerular filtration threshold of 45,000 Da [19]. Polymers of higher molecular weights remain in the circulation indefinitely as they are non-degradable [52,130]. Intravenous application of a range of HPMA polymers showed clearance from the blood stream to be fairly rapid but dependent on molecular weight. Accumulation in tissue *e.g.* spleen and liver, is only apparent with polymers of 778,000 Da or above. Thus, most interest has been focussed on polymers of molecular weight less than 20,000 Da to ensure glomerular filtration.

As has been discussed in Chapter 1, the distribution of HPMA co-polymers *in vivo* can be altered easily by the incorporation of non-specific hydrophobic groups [34] and charge or by the use of specific targeting residues [45,63]. In tumour-bearing animals, conjugation of drugs to HPMA co-polymers results in an increased concentration of the drug in tumours, suggesting that the co-polymer is tumour tropic [81,82].

### 2.6.3 Degradation

Obviously, due to their synthetic nature, co-polymers of HPMA cannot be degraded by enzymes in the blood stream or lysosome. This could lead to an unacceptable accumulation of the polymer within the body. Hence, with these linear polymers, the molecular weight must be kept below the renal threshold, potentially reducing the tumour tropic effect. Accumulation of the polymers can be avoided by the use of soluble cross-linked polymers. By careful choice of the cross-link, spacers can be prepared which only degrade within the lysosome *i.e.* after delivery of the drug. The resulting low molecular weight polymers can be excreted in the urine [131,132].

An example of a cross-linked HPMA co-polymer is shown in Figure 2.7 [131,132] . The cross-linking reaction is designed to occur below the gel point in order to produce a soluble rather than an insoluble polymer. This reaction has to take place at low concentrations owing to the low solubility of the reactants. However, this could result in the formation of cyclised structures with the amine reacting twice with the same polymer chain. This can be avoided by cross-linking the polymer through short diamines such as ethane-1,2-diamine. This, however, could lead to decreased enzymatic degradability. Cyclisation can be more easily overcome by employing a two-step reaction. Treatment of the polymer with a large excess of the diamine will minimise cyclisation and maximise the formation of free amines. In a second step, the free amino groups can then be allowed to react with more polymer starting material [123]. Two-stage cross-linking also allows the formation of very high molecular weight products [128].

Initially, model studies on the degradation of peptide sequences between the bis-amine and polymer were performed. Amino acid sequences were prepared which showed similar specificity as side-chains for the enzymes chymotrypsin [128,131], trypsin [133] and papain [134]. In all cases, degradation was reduced in comparison to the same chains attached to the linear polymers. This could be due to either decreased access of the enzyme to the bond to be cleaved or to conformational constraints of the amino acid sequence as it is tethered at both ends.

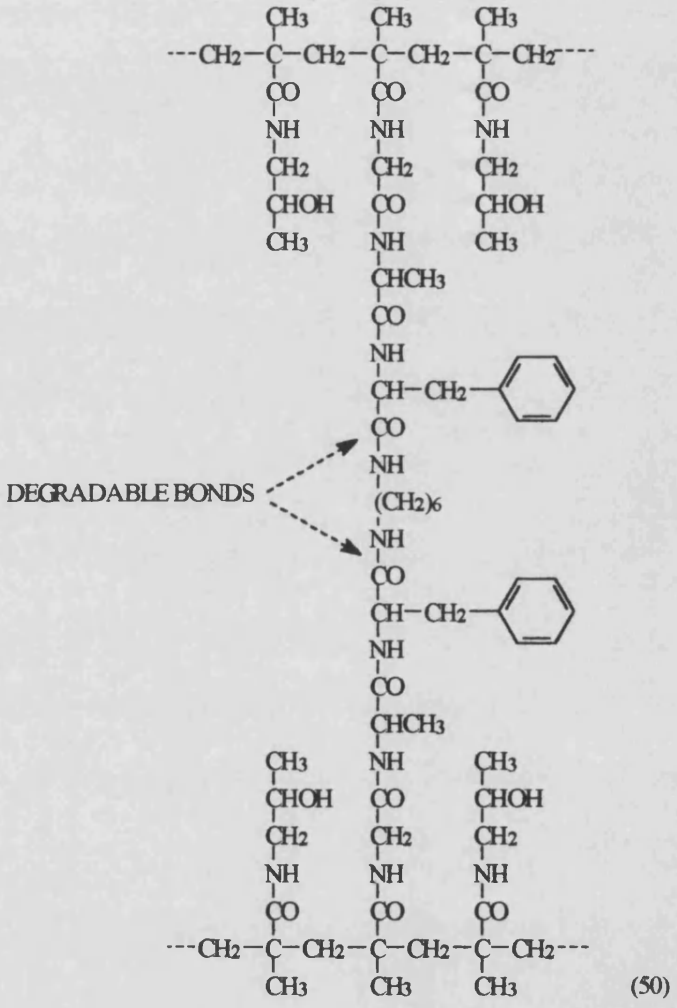



Figure 2.7

This decrease in degradability was especially pronounced with cross-linked polymers with either a two amino acid peptide sequence or a short diamine [131,134].

Cross-linked polymers have been evaluated *in vitro* for lysosomal degradation using isolated cathepsin B. Degradation was again highest with long amino acid chains or with a long bis-amine [69]. In a further study, cross-linked polymers with the amino acid sequence GlyGlyPheTyr were degraded easily by rat lysosomal enzymes but were resistant to degradation in plasma. Preliminary experiments with the same polymer *in vivo* in rats confirmed degradation takes place as low molecular weight chains were excreted [135,136].

This approach has not been investigated further in the development of intravenous agents for the treatment of cancer, although HPMA cross-linked polymers formed above the gel point are being evaluated for colon-specific drug release [137].

### 2.6.4 Immunogenicity

The immune response to both HPMA and co-polymers of HPMA has been thoroughly investigated by Rihova [101]. Injection of the homopolymer does not appear to result in an immune response in mice as no antibody titre is measurable. Incorporation of peptidyl side chains, however, does result in the production of antibodies. This is a weak reaction, the titre is considerably lower than for a model immunogen, bovine gamma globulin, and the antibodies are of the weak IgM class [138]. Injection of a wide range of doses of the co-polymers demonstrated a tolerance reaction; the antibody response to most co-polymers was higher for a 10 μg/kg dose than for a 100 μg/kg dose. The structure of the peptide also had an effect on the antibody titre but no structure-activity relationships could be evaluated [138].

Interestingly, using one peptide spacer at different substitution levels did not affect the number of antibodies raised. This suggests that the presence of only one or two epitopes, as in the 1 mol% conjugate, can elicit an antibody response [138].

The specificity of the antibodies has also been investigated in cross-reactivity reactions. These showed that the majority of antibodies formed were directed to the peptide sequence and therefore could not cross-react but some were raised against the polymer backbone, most probably against the hydroxypropyl unit. This suggests that, although antibodies are not raised against the homopolymer, once the entire molecule becomes immunogenic due to the peptide chain, antibodies are raised against all parts of the molecule [138]. Similar results were obtained with co-polymers bearing adriamycin. A low antibody response were seen at all doses and was unaffected by the incorporation of galactosamine targeting residues [130].

The effect of incorporation of known haptens has also been investigated. Again, the majority of antibodies were raised against the hapten. With this system, the effect of molecular weight on immunogenicity was evaluated. More antibodies were raised to the hapten with a co-polymer of molecular weight 200,000 Da than for one of 5,000 Da [101].

The effect of pre-immunisation on the efficacy of a daunomycin HPMA co-polymer has also been evaluated. Pre-immunization can either give no effect, an adverse toxic affect or result in loss of activity of a drug. In this system, pre-immunization over 35 days prior to inoculation of the mice with L1210 cells resulted in no loss of activity of the conjugate [139].

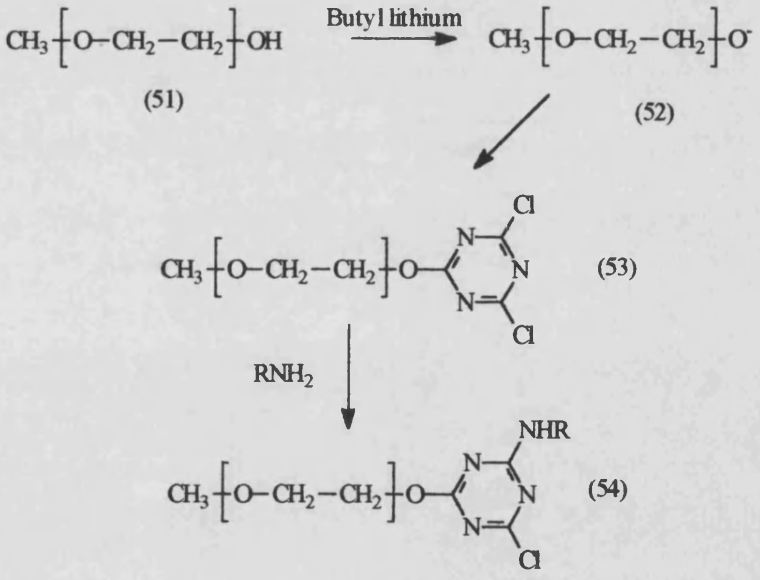
## 2.7 Poly(ethylene glycol) PEG

Poly(ethylene glycol) is a non-toxic, non-immunogenic, highly water-soluble linear polymer [140]. It has mainly been used medically in the modification of therapeutic proteins. Modification of proteins with PEG leads to a decrease in the toxicity and immunogenicity of the protein, whilst increasing both the water solubility and plasma half-life [140,141,142]. These properties suggest that conjugation of a drug to PEG or PEG co-polymers would provide an ideal non-toxic macromolecular pro-drug with enhanced plasma lifetime and potentially increased uptake into tumours.

2.7.1 Activation

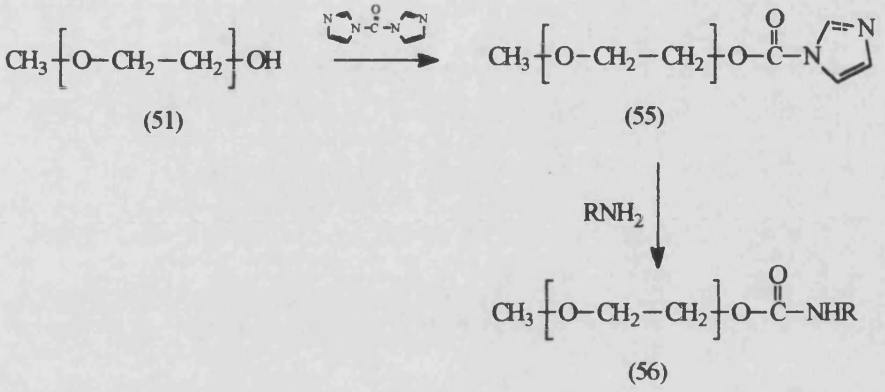
Much research has been directed towards the preparation of activated PEG for attachment to proteins. The coupling reaction in this system is usually between PEG monomethyl ester and the ε-amino group of protein lysine residues [140]. Therefore, most active derivatives are based on carboxylic acid-type groups. For the conjugation of drug or the formation of block co-polymers, amine residues may be required. These can be provided by treatment of the carboxyl-activated PEG with bis-amine or by direct conversion of PEG to PEG-amine [143,144].

The activation of PEG by cyanuric chloride is one of the oldest and most widely used methods for protein coupling. The original method of Abuchowski and Davis has been modified by Harris [145] to enable complete conversion within two hours, through the use of alkoxides (Scheme 2.13). Coupling of proteins using this method has been linked to inactivation of the protein [141,146], cross-linking and non-specific coupling to OH and SH residues [147]. Thus, this method has been overtaken by more recent advances.



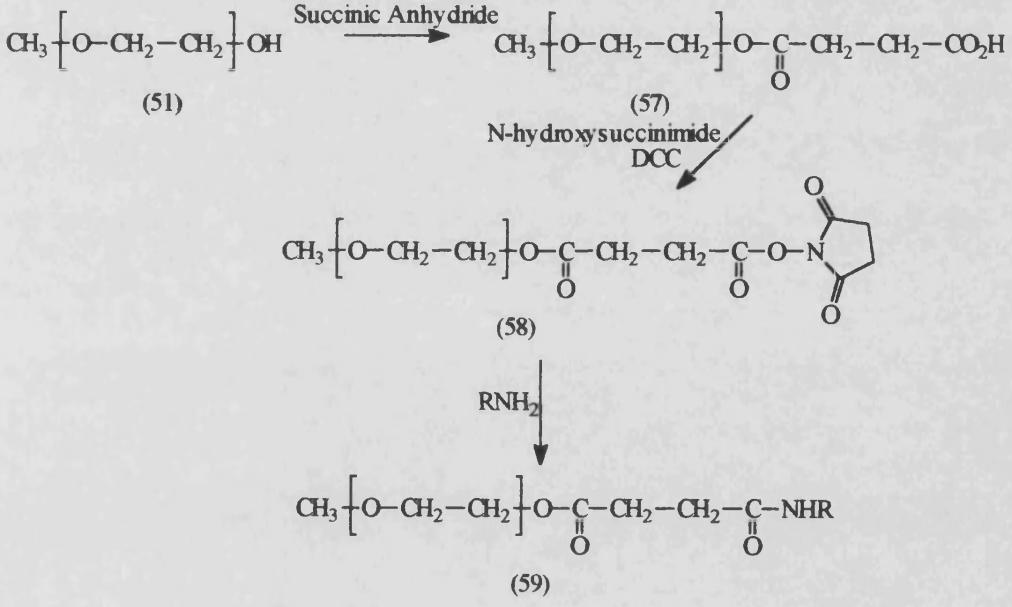

Scheme 2.13

Activation using CDI (Scheme 2.14) is effective and does not lead to cross-linking. However, the coupling reaction is slow and can take up to 72 hours. This method, therefore, is of little use in co-polymerisation reactions [147].



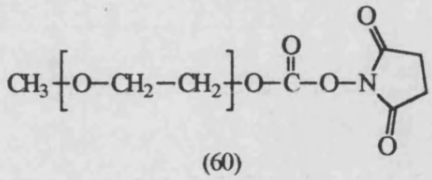

Scheme 2.14

Treatment of PEG with succinic anhydride generates a carboxylic acid group which can then be activated *via* a DCC coupling to form the N-hydroxysuccinimide ester (Scheme 2.15) [146,147]. These derivatives react quickly with amines [148] but the resulting product can be unstable due to hydrolysis of the ester linkage[147].




Scheme 2.15

An alternative approach, developed by Zalipsky, allows the formation of a N-hydroxy-succinimide-activated PEG without hydrolytic instability. Treatment of PEG with phosgene followed by N-hydroxysuccinimide results in a stable urethane derivative between an amine drug and PEG (Figure 2.8) [148]

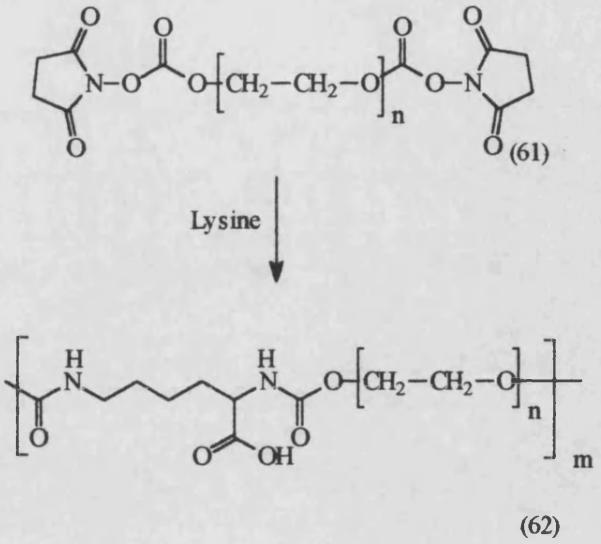


(60)

Figure 2.8

These are only the main methods used. Activation and coupling have also been achieved by reductive amination with PEG aldehyde, by formation of glycidyl ethers with epichlorohydrin [149,150] and the formation of tresylates (2,2,2-trifluoroethanesulphonates) [147, 151]

A number of co-polymers of PEG have been prepared for use in drug delivery. Zalipsky [148,152] utilised the N-hydroxysuccinimide method to prepare a co-polymer of PEG and lysine with the amino acid linked through the α- and ε-amino groups (Scheme 2.16). The degree of polymerisation could be easily altered by varying the reaction time and concentration of the reactants. A polymer of molecular weight of 170,000 Da was achieved in a two hour polymerisation from PEG of mean molecular weight 2,000 Da. The co-polymer produced has pendant carboxylic acid groups that can be easily activated as the azides or active esters [148].




(61)

(62)

Scheme 2.16

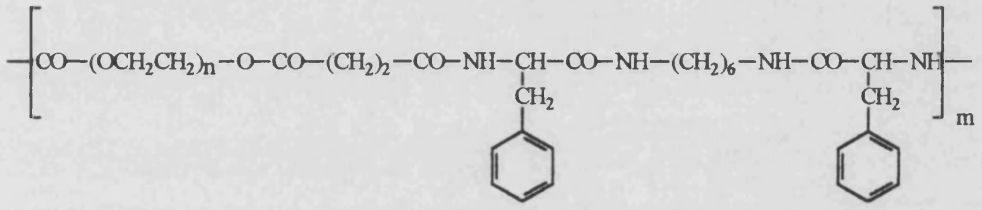
An AB Block co-polymer of monomethyl PEG with poly(aspartic acid) has also been prepared. In this case, an amine derivative of PEG acts as an initiator for the N-carboxyanhydride polymerisation of β-benzyl-protected aspartic acid. [153]

2.7.2 Degradation

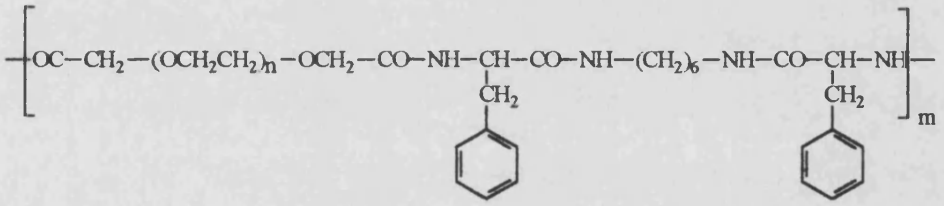
PEG as well as being chemically inert, is not susceptible to biodegradation. Thus, if high molecular weight co-polymers are to be used for macromolecular pro-drug production, enzymatically degradable units must be introduced to the structure. Obviously, in a co-polymer of PEG and poly(aspartic acid), the aspartic acid unit will be degradable, allowing breakdown of the polymer [153]. Unfortunately, this degradation would not be specific to the lysosomal compartment. However, by developing particular small peptide linkages, it may be possible to produce a selectively degradable co-polymer.

Ulbrich *et al.* [78] investigated the degradability of PEG units linked *via* diamines incorporating one amino acid, Phe. Three polymers were synthesised, one containing both enzymatically and hydrolytically degradable bonds (Polymer A) and two containing enzyme-sensitive bonds (Polymers B and C) (Figure 2.9). These workers

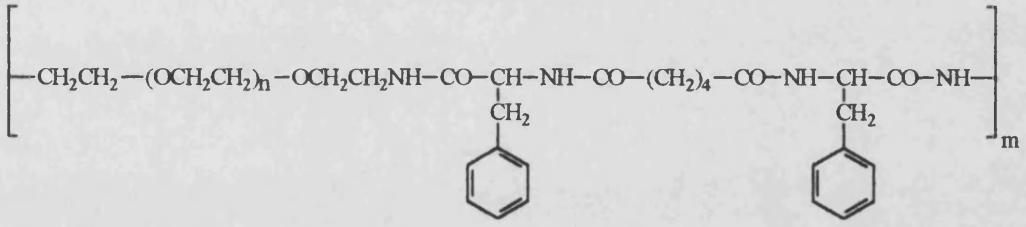
were able to demonstrate the cleavage of all three polymers by chymotrypsin, however only polymer A was degraded hydrolytically. With polymers B and C, the rate of degradation depended on the orientation of the peptide bond with respect to the PEG chain. In polymer B, more favourable enzyme-substrate interactions are provided, where PEG is in $P_2$ to $P_4$ than in polymer C where these positions are occupied by the bis-amine. Thus, the degradation of Polymer B is faster than Polymer C. These results demonstrate the ease of producing degradable PEG co-polymers [78]. However, before these polymers can be used as drug carriers either an amino acid with functionality or a modified bis amine must be introduced to allow attachment of a drug molecule.



POLYMER A (63)



POLYMER B (64)



POLYMER C (65)

Figure 2.9

### 2.7.3 *In vivo* Distribution

PEG is uncharged and hydrophilic and, therefore, does not interact easily with body components. An example of this is the reduced uptake of microspheres into the reticulo-endothelial system when they are coated with PEG [153].

Studies [154] of the body distribution of PEG injected intravenously to mice show that the half-life is dependent on molecular weight and can vary between minutes and days. Urinary clearance was dependent on molecular weight and a glomerular filtration threshold was calculated to be 30,000 Da. Uptake by other organs was unrelated to the molecular weight of the polymer; appreciable accumulation was only seen in the kidney and liver. This accumulation was thought to be due to uptake by macrophages and Kupffer cells.

### 2.7.4. Immunogenicity and Toxicity

PEG has low toxicity. Studies in dogs have shown no toxic or cumulative effects even at the high dose of 90 mg/kg a day [140]. Studies in rats have confirmed these results, administration of a 16% w/w oral dose of PEG had no adverse effects. There have been occasional reports of toxicity in man on oral and topical administration. However these reactions are not seen with intravenous application [147].

PEG is also non-immunogenic. Antibodies were not raised to PEG of molecular weights up to 100,000 Da in rabbits, even in the presence of complete Freund's adjuvant. Investigations on the immunogenicity of PEG-protein conjugates have demonstrated a decrease in the antigenicity of the protein. Proteins are highly antigenic due to their heterogeneity. The decrease in immunogenicity, measured by decreased antibody titre, is thought to be due to a shielding effect of the PEG chain, preventing recognition of the haptens [101,140,146].

When antibodies are raised to PEG-protein conjugates, a portion of the antibodies are raised against the PEG chain. Injection of PEG-allergens to humans does result in the

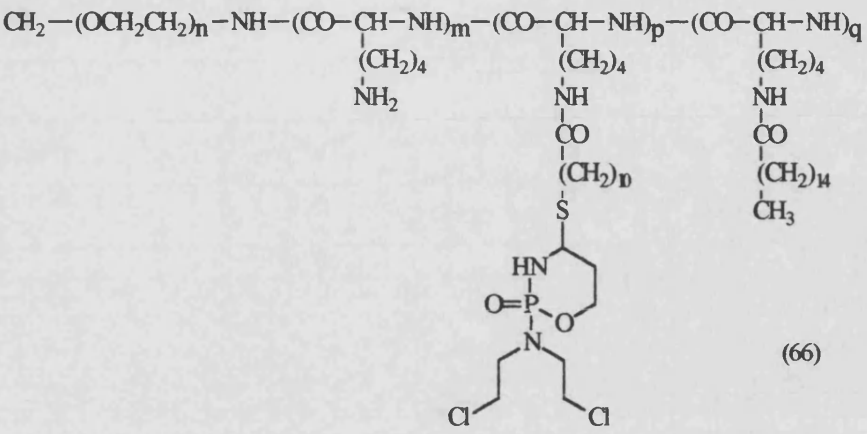
formation of PEG antibodies in 50% of the population but these are of the weak IgM type. In fact, two years after administration, no circulating antibodies to PEG can be seen. This response, therefore, is not thought to be of clinical significance [140].

The shielding property of PEG may also prove beneficial with PEG block copolymers bearing drugs. Many drugs are very active haptens, so coupling with PEG co-polymers may reduce their immunogenicity. The micellar structure formed by some AB block co-polymers of PEG are also highly unlikely to be immunogenic.

### 2.7.5 Micelles

Initial studies by Pratten *et al.* on block co-polymers of PEG and Poly(Lys) substituted with palmitoyl groups gave surprising results [40]. It was expected that pinocytic uptake of this polymer would be high due to hydrophobic interactions between the palmitoyl groups and the cell surface. However, no improvement over PEG was seen. It was suggested that the block co-polymer forms a micellar structure in which the palmitoyl groups are in a central core and cannot interact with the cell surface.

This system was further investigated by Ringsdorf *et al.*, [6] with a drug moiety, an inactive cyclophosphamide, attached to the palmitoyl group. The drug is usually rapidly hydrolysed to an active derivative. However, when attached to the polymer, this hydrolysis was slower. The cross-linking of DNA due to the drug was evaluated *in vivo* in L1210 cells. Maximal cross-linking was achieved considerably later with the conjugate than with free drug. It was suggested that the drug was within a micelle core and was only released after cellular uptake [6].(Figure 2.10)



(66)

Figure 2.10

More recently, Yokoyama *et al.* [155,156] have consciously developed a block copolymer pro drug system that spontaneously forms micelles in solution. The system is based on a co-polymer of PEG and poly(Asp), where drug molecules such as adriamycin can be attached to the aspartic carboxylic functions. The co-polymer is easily produced. PEG acts as the initiator for the polymerisation of the aspartic unit and, following de-protection of the polymer, adriamycin can be introduced via an EDC coupling [155,156].

Incorporation of a drug onto a macromolecule is usually limited by the solubility of the conjugate; as the level of drug increases, precipitation can occur. With these block polymers, however, 50 mol % of drug can be incorporated [156,157]. This property is dependent on the solubilizing action of PEG, as similar incorporations cannot be achieved with poly(Asp) [158]. Analysis of these co-polymers gave apparent molecular weights of over 30,000 Da, where the single chain molecular weight is 9,000 Da. This suggests that an aggregation process has occurred, *i.e.* a micelle has been formed. This is confirmed as these structures are broken on treatment with a surfactant [153].

It has been suggested that single polymer chains exist in an equilibrium with the micelles and that drug release is only achieved from the single chain [158]. The micelle size can be altered within tight limits by altering the molecular weight of the PEG chain. However, altering either the length of the poly(Asp) chain or the content of incorporated drug does not affect the size of the micelle [158].

These systems have been extensively investigated both for their distribution *in vivo* and cytotoxicity [157]. In an distribution study *in vivo*, the existence of micelles was confirmed. No urinary clearance was measured for two conjugates based on PEG 5,000 and 12,000 despite the fact that their molecular weights are below the glomerular filtration threshold. The conjugates remained in the blood stream for a prolonged period and were passively accumulated into the tumour. After 24 hours a concentration of 10% dose/gram of tissue was seen for the tumour, whereas for free drug the best concentration was 0.9%. Coupled with this, the conjugate displays decreased accumulation in the heart, a major organ for toxicity, in comparison to free drug [157,158]. The activity in mice with P388 leukaemia has also been evaluated. The conjugates displayed increased activity and decreased toxicity when compared to the free drug [158].

These workers have also attached an antibody to this system and demonstrated that the micellar structure is lost. In this situation, the single polymer chains form an ideal conformational state in which the disulphide linkage to the antibody is protected by the aspartic acid residues [153,155]. Micelle formation is only possible with block co-polymers of an AB structure. However, it is thought that, in more complicated co-polymers, the molecule will arrange itself into the most suitable conformational state. With PEG co-polymers, it is thought that the PEG will be exposed to the circulation and hydrophobic units will be protected, as is the case with PEG proteins [153].

### 2.7.6 Macromolecular pro-drugs based on PEG

An interesting approach to the use of PEG as a macromolecular carrier has recently been proposed [159]. In this case, one PEG chain is coupled to one drug molecule e.g. adriamycin, and one targeting group, lactose. This conjugate was active *in vitro* in both leukaemia and hepatoma cell lines. Despite this efficacy, this system cannot be considered ideal as a large number of conjugates would have to be delivered to the cell rather than one conjugate bearing many drug molecules.

Nathan *et al.* [148,152] have investigated a co-polymer of PEG and lysine as a carrier for the antibacterials; cephradine and penicillin V. In this approach, the pendant carboxylic acid group of the amino acid was successfully converted to other reactive groups, such as amino and hydroxyl, to enable the coupling of a variety of compounds. Both biodegradable and non biodegradable conjugates were prepared, a number of which also incorporated a spacer.

## 2.8 Conclusion

Thus, although many macromolecules have been investigated for the preparation of macromolecular pro-drugs, none is ideal. At present, the system with the greatest potential is pro-drugs based on co-polymers of hydroxypropylmethacrylamide (HPMA). Macromolecular pro-drugs formed from this polymer are at present undergoing clinical trial. A prospect for the future is the development of macromolecular pro-drugs based on poly(ethylene glycol) (PEG) and degradable linkages.

# CHAPTER THREE

# CONTRAST AGENTS IN MAGNETIC RESONANCE IMAGING

## 3.1 Introduction

The majority of research on macromolecular carriers has involved the investigation of macromolecular pro-drugs for the treatment of cancer. However, almost any drug can be attached to a carrier to allow targeting or to increase blood circulation time. Macromolecules can also be used in the imaging of tumours and other diseases. A range of macromolecular contrast agents have been produced for use in magnetic resonance imaging (MRI). These are successful not only in the imaging of tumours and inflammation but as blood pool contrast agents allow investigation of perfusion and occlusion of the vascular system. The basis of contrast enhancement of MR images and contrast agents are considered in this chapter.

## 3.2 Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) is based on detecting the NMR signals of water protons within the body. Tissues contain varying amounts of water and this is the basis of contrast between organs. This is enhanced by using a gradient magnetic field. The protons then resonate at a different frequency depending on the position within the field. This allows spatial information to be combined with the resonance frequencies and transformed into an image of the organs within the body [160,161]

When a magnetic field is applied to a proton, the induced magnetism is aligned along the Z axis. When a radio frequency pulse is applied, this magnetic vector moves away from the axis and then returns or 'relaxes' back to the axis. The time taken for this relaxation is $T_1$, the spin-lattice (longitudinal) relaxation time. A component of the magnetic field lies perpendicular to the Z axis; this is also perturbed by application of

a pulse and relaxes back with a relaxation time $T_2$, the spin-spin (transverse) relaxation time. So, in MRI, pulses are applied rapidly to prepare images that are based on the different relaxation rates of water protons [160].

In some cases, in normal, non-enhanced tissue, the properties of two tissues are not sufficiently different to allow imaging of lesions or tumours. In these cases, a contrast enhancement agent can be used. These can either alter the spin density of a tissue or the relaxation times of the protons within the tissue. Spin density is a measure of the number of protons in an area. By using gases, such as $CO_2$, and perfluorocarbons, which have no hydrogen atoms, the total spin density within the area can be reduced giving a decrease in signal intensity [160,163].

However, most interest has been focussed on altering the $T_1$ and $T_2$ times of tissues. Tissues with short $T_1$ give brighter images than those with long $T_1$ as the majority of the magnetism is aligned along the Z axis at a given time. In contrast, tissues with short $T_2$ values have decreased signal intensity and are seen in images as dark areas. The values of $T_1$ and $T_2$ can be altered *in vitro* and *in vivo* by the addition of compounds with magnetic properties [160,162].

### 3.2.1 Diamagnetism

Most molecules are diamagnetic; that is, they have no magnetic dipole as they contain no unpaired electrons. In an applied magnetic field, they do exhibit a small net magnetism which is aligned antiparallel to the applied field. This small local field exerts only a minor effect on the water protons of a tissue. Hence, addition of a diamagnetic ion does not result in enhanced images [164].

### 3.2.2. Paramagnetism

Paramagnetism occurs when an ion or molecule contains unpaired electrons. Although there is no net magnetism without application of an external field, once an external field is applied orientation of the unpaired electrons results in the formation of a

strong local magnetic field. Paramagnetism is prevalent in transition metals, where there is preferential filling of the 4s level giving rise to a number of unpaired electrons in the 3d level, and the lanthanides. Thus $Mn^{2+}$ with five unpaired electrons and $Gd^{3+}$ with seven unpaired electrons are effective paramagnetic ions [164].

If a paramagnetic ion is introduced to the body, it can interact with the proton nuclei of water molecules to reduce both the $T_1$ and $T_2$ relaxation times. The ability of the ion to alter the times depends upon the ease of approach of the proton to the paramagnetic centre and is termed relaxivity. This property depends on the magnetic moment of the ion which is itself related to the number of unpaired electrons. Thus $Mn^{2+}$, $Gd^{3+}$ and $Fe^{3+}$ ions have high relaxivities due to their large magnetic moments [162].

The type of interaction can be inner sphere in which a molecule of water binds directly to the primary co-ordination sphere of the metal ion from which it can exchange with the bulk water. Alternatively outer sphere interactions, where there is no direct binding, can occur. The relaxivity is a combination of these effects, although outer sphere mechanisms are only of interest with chelated ions [160]

Relaxivity is also dependant on the correlation time, which is a term describing the likelihood of an interaction occurring between the unpaired electron and a water molecule. The correlation time is the sum of three correlation times; the rotational $T_R$, the electron spin relaxation $T_S$ and chemical exchange $T_M$ [162].

$$1/T_C = 1/T_R + 1/T_S + 1/T_M$$

Increasing the rotational correlation time, by slowing molecular motion of a paramagnetic ion can lead to increased relaxivity. This has been demonstrated by the attachment of paramagnetic ions to slow-tumbling protein macromolecules. Careful choice of paramagnetic ion can also enhance relaxivity; paramagnetic compounds based on Mn and Gd and nitroxides have long electron spin relaxation times and high

relaxivity, whereas Dy has a faster electron spin relaxation time and lower relaxivity [162].

Paramagnetics alter both $T_1$ and $T_2$. Reduction in $T_1$ leads to an enhanced signal intensity, whereas reduction in $T_2$ leads to a loss of image intensity. Thus, a balance must be found between these two effects to achieve contrast enhancement. At low field strengths, the decrease in $T_1$ dominates the relationship and enhanced images are seen, at higher fields the $T_2$ effects dominate and signal enhancement is reduced. The concentration of paramagnetic ions can also alter the imaging effect at high concentrations as $T_2$ effects dominate. Thus, in producing images, it is important to alter the applied magnetic field strength to provide either $T_1$ or $T_2$ weighted images. With paramagnetic ions and chelates, the majority of images are produced with positive enhancement $T_1$ weighted scans [160,161,162,164].

### 3.2.3 Ferromagnetism

Ferromagnets are permanent magnets. This is due to a property of a group of atoms or molecules in a solid crystal rather than of a single ion. When an external field is applied, the molecules arrange to produce a magnet which remains even when the field is removed. However, if the size of a multidomain ferromagnet is reduced to less than 350 Å, a single domain particle can be formed. These particles have slightly different properties, although they are rapidly magnetised in an external field like ferromagnets, when the field is removed they behave as paramagnets and do not retain the induced magnetism. These particles are known as superparamagnets. The most commonly used superparamagnetic contrast agent is based on small particles of magnetite, a naturally occurring iron oxide $Fe_3O_4$. Superparamagnetics, unlike paramagnetics, have a monophasic action on relaxation times. Thus $T_1$ relaxation times are barely affected by the administration of a superparamagnet but $T_2$ is greatly increased allowing negative enhancement images [164].

Thus it is possible to design contrast enhancement agents for the imaging of disease states by altering the magnetic environment in particular tissues. Initially, free

paramagnetic ions were used to produce this effect but these have now been developed further, with the production of metal chelates and the use of pharmaceutical delivery forms, to achieve excellent organ specific imaging.

## 3.3 Metal Ions

As early as 1948, paramagnetic ions, such as $Fe^{3+}$ in ferric nitrate, were used to enhance the relaxivity of water protons [160]. This phenomenon was exploited in gastro-intestinal imaging, but the free ferric ions caused irritation of the gastro-intestinal tract. Further studies using $Mn^{2+}$ enabled the imaging of myocardium in dogs. This cation localises in healthy myocardium but cannot be taken up into infarcted regions. Thus imaging of infarction was possible. These studies showed that the degree of relaxation enhancement was directly proportional to the concentration of paramagnetic ion in the organ. Although these results were promising, the free paramagnetic $Mn^{2+}$ ion is highly toxic so cannot be used in MRI. It is taken up by calcium transport mechanisms in the body, resulting in cardiovascular toxicity [162].

## 3.4 Metal Complexes

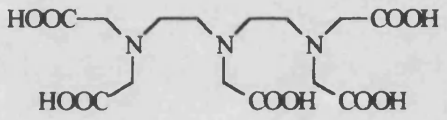
Complexes of paramagnetic ions with co-ordinate ligands have been widely tested as contrast enhancement agents. Although the relaxivity of most of these complexes is reduced in comparison to the free ion, owing to decreased inner sphere binding of water, the toxicity of the paramagnetic ion is also reduced. Thus a larger dose can be given overcoming the reduced relaxivity [162]. The main paramagnetic ions used in these systems are $Gd^{3+}$, $Mn^{2+}$ and $Fe^{3+}$ although chromium EDTA complexes were used in initial studies [165,166,167,168]. Both cyclic and acyclic ligands have been developed, although at present only one compound, an acyclic compound, is in general clinical use.

3.4.1 Acyclic Ligands

The original acyclic ligands were developed from EDTA. EDTA has been used since the early 1950s as a detoxifying agent for iron overload. Since then, it has been shown that modification of the ligand structure can result in enhanced contrast in a variety of tissues and coupling to targeting moieties can achieve cell line targeting [162].

3.4.1.1 Gd-DTPA

The dimeglume salt of Gd-DTPA (Figure 3.1) has been used in the clinic for over ten years in the imaging of brain and spinal tumours. Model studies in rats [169] demonstrated the potential of contrast enhancement imaging of radiation damage in the brain. These studies were rapidly advanced into human trials, where cerebral tumours were more accurately contrasted with oedema and normal tissue using the conjugate [170].




(67)

Figure 3.1 : DTPA

Gd-DTPA exerts its effect in an extracellular manner and undergoes rapid renal clearance; it is therefore a useful contrast enhancement for the assessment of renal function. In studies in rats [169], enhancement of the kidney parenchyma was achieved five minutes post injection and was retained for over 90 minutes. The complex has also been used in angiographic studies [171,172], to determine neurological conditions, vascular occlusion and atherosclerotic disease. Imaging of small vessels not seen without a contrast agent was possible with Gd-DTPA.

Despite these promising results, Gd-DTPA is not a universal contrast agent for all disease states. Imaging of liver disease and tumours is not possible due to rapid

exchange between the normal and diseased tissue leading to the entire region showing enhanced contrast [173].

Targeting of a DTPA complex has however been achieved, with a radioactive chelate, to malignant melanoma. Two DTPA $^{111}In$ chelates were conjugated to MSH. This allowed targeting to the α-MSH receptors on malignant cells and gave excellent radiographic imaging. This technology could be easily transferred to the delivery of paramagnetic ions [174]. Phosphonate chelates of $Gd^{3+}$ have also been prepared to enable targeting to myocardial cells [172].

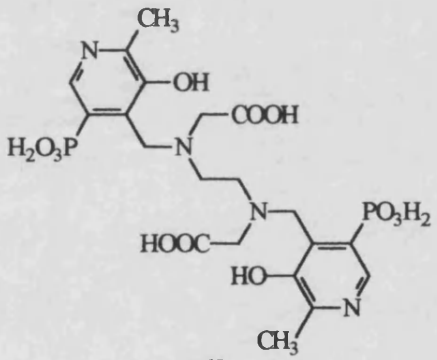
Gd-DTPA is of low toxicity. A small amount of demetallation does occur but is not significant and the complex is extremely safe in the normal dosage range [162]. However, the charged complex becomes more toxic at higher doses owing to increased osmolality. More recently, non-ionic complexes derived from DTPA have been prepared. These have comparable molar relaxivities to Gd-DTPA but reduced osmolality and hence can be used over a larger dosage range [175]. The most commonly used derivative is Gd-DTPA bis(methylamide) or Gadodiamide injection [176].

3.4.1.2 Mn-DPDP

As Gd-DTPA cannot be used successfully in the imaging of liver carcinoma, many attempts have been made to prepare similar chelates which are in some way targeted to the liver. Manganese (II) N,N'-dipyridoxalethylenediamine-N,N'-diacetate-5,5'-bis(phosphate) (Mn-DPDP) (Figure 3.2) is designed to be targeted to hepatocytes through the pyridoxal-5'-phosphate moieties. This co-enzyme is taken up by a membrane transport system in hepatocytes as is the chelate [177]. It is therefore possible to distinguish between functioning and non-functioning hepatocytes, that is, between normal and diseased tissues [178,179]. Thus, negative contrast enhancement is achieved, the normal cells showing hyperintense relative to the diseased cells.

In healthy subjects, enhancement of liver parenchyma was achieved after one minute and persisted for 30 minutes. Bile imaging was also possible after 15 minutes due to hepatobiliary excretion of the chelate [180]. Few side effects were seen with this compound, although the majority of subjects did experience facial flushing.

Pre-clinical studies in tumour bearing rats [177] demonstrated a small accumulation of the chelate in the liver and initial images gave no contrast between normal and tumour tissue. After 30 minutes however, there is enhanced tumour to liver ratio, presumably due to decreased excretion from the tumour cells. Obviously, the degree of uptake of the chelate by diseased cells depends on the level of normal hepatocyte function retained. Thus it is possible to achieve differential diagnosis of liver tumours [178,181]. Metastatic liver disease and well-differentiated tumours have no uptake and thus are easily negatively imaged, whereas non differentiated tumours can only be visualised by a positive enhancement about 24 hours after administration.

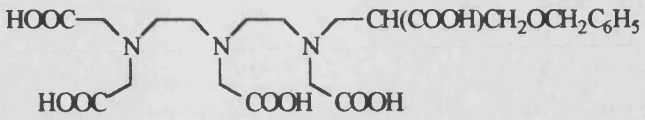



(68)

Figure 3.2 : DPDP

3.4.1.3 Gd-BOPTA

New ligands for gadolinium have also been prepared to allow liver targeting. Gd-BOPTA is a derivative of DTPA with a benzyloxymethyl group in the backbone (Figure 3.3). It is intended that this should be taken up by the anionic hepatocyte receptor which takes up bromosulphophthalein [162]. This complex has a high proportion of biliary excretion and is accumulated in liver in rats, in which organ normal cells can easily be imaged [173]. This leads to negative imaging of tumours [182,183].

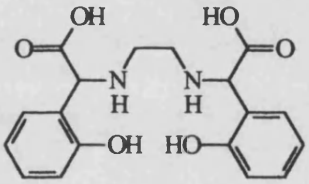


(69)

Figure 3.3 : BOPTA

3.4.1.4 Fe-EHPG

A lipophilic chelate of iron has been produced (Figure 3.4) [184]. This is designed to be taken up into rat liver cells through non specific hydrophobic interactions. Good localisation in the liver and bile was achieved. However, this chelate has lower relaxivity than other chelates and large doses are required; this is a problem as some toxicity has been seen [162]. Studies in mice demonstrated increased signal intensity of both the liver and bile in healthy animals and enhanced visualisation of intrahepatic implanted tumours and a metastatic model [185]. Increasing the lipophilicity can lead to decreased liver uptake as the chelate binds to serum proteins; therefore, the chloro derivative is most widely used owing to its favourable balance of protein binding and liver uptake [160].



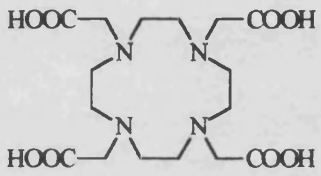
(70)

Figure 3.4 : EHPG

### 3.4.2 Cyclic Ligands

#### 3.4.2.1 Macrocycles

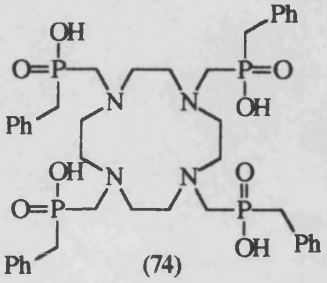
In the search for even more effective chelates, macrocyclic ligands have been produced. Although these complexes take up paramagnetic ions slowly, they exhibit increased stability once the ion is bound and hence reduced risk of toxicity due to leaching. The major ligand that has been evaluated is Gd-DOTA [186] (gadolinium tetraazacyclododecanotetraacetic acid) (Figure 3.5). This complex has similar relaxivity to Gd-DTPA and similar biodistibution but is less toxic [187,188]. It is also has a more enhanced relaxivity at low field strengths due to the effect of symmetry on the electron relaxation time.




(73)

Figure 3.5 :DOTA

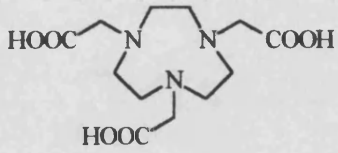
As with DTPA, non ionic complexes have been made such as DO3A [162]. Azaphosphonic acid derivatives have also been produced which can be either anionic, cationic or neutral and suitably functionalised for the attachment of targeting moieties [161]. These complexes (Figure 3.6) are targeted to the liver and bile. Radiolabelled Gd complexes of the tetraphosphonate **(74)** have been shown to clear more slowly from tumour tissue than normal tissue allowing a degree of specificity [161].




(74)

Figure 3.6

Tumour targeting has also been achieved with indium-labelled NOTA complexes. These compounds accumulate in some skin cancers. However, NOTA is not a good ligand for paramagnetic ions [161,162]. Cyclam complexes of paramagnetic Mn(III) and Cu(II) have also been produced (Figure 3.7). Mn (III) cyclam is a highly stable complex with comparable relaxivity to Gd-DTPA and localises as efficiently as Fe-EHPG in the liver [189].




(75)

Figure 3.7 : NOTA

3.4.2.2 Metalloporphyrins

Metalloporphyrins are endogenous metal-binding ligands. Although they are of complex chemical structure, they are of increasing interest owing to selective uptake by some tumours [162]. The selective uptake of porphyrins into tumour cells was first noted in the 1920s. More recently, this property has been exploited in photodynamic therapy [190]. Some derivatives of haematoporphyrin can be localised to tumours and act as photosensitisers for conversion of triplet oxygen to cytotoxic singlet oxygen. [190].

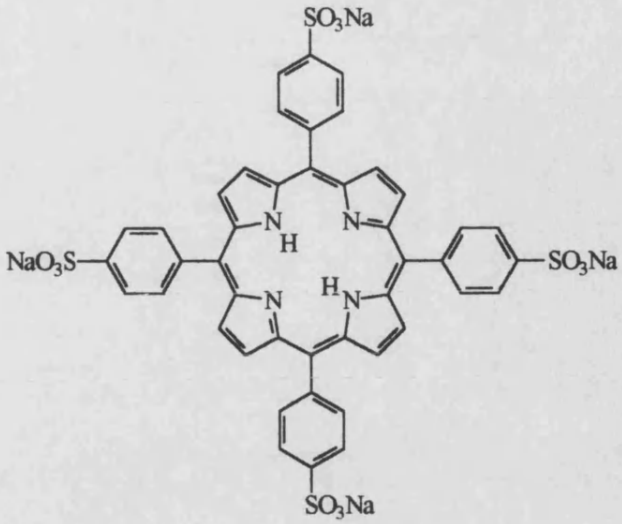
Incorporation of a paramagnetic ion into a porphyrin could, therefore, result in a higher tumour concentration of a contrast agent. Accumulation of metal-bearing polymers in tumour cells has been demonstrated by many workers, although some have suggested that the tumour selectivity of porphyrins can be reduced by metallation. The mechanism by which tumour selectivity occurs remains unknown [191]. It has been suggested that the porphyrin could be trapped in cells by enzymic alteration or be compartmentalised into specific regions in the cell. Alternatively, it is possible that the porphyrin binds to proteins or fibrous tissue, or is taken up into cells

by specific transport mechanisms. Studies on the retention of both TPPS and Mn-TPPS in human breast cancer cell lines confirmed that the most likely mechanism is selective uptake *via* a porter for TPPS [191].

Although porphyrins can chelate numerous metal ions, the most useful in terms of imaging is manganese. Gadolinium is too large an ion to form a stable complex so is easily leached and iron loses its paramagnetism at pH>6 when chelated into porphyrins [162]. Manganese fits easily into the porphyrin and these complexes have surprisingly high relaxivities of over 10 $mM^{-1}s^{-1}$. Although direct comparisons cannot be made with Mn (III) ions as they are unstable in the circulation, this relaxivity is three times higher than that of $MnCl_2$. It has been suggested that the increased relaxivity is due to closer interactions between the paramagnetic ion and the water protons or to symmetry effects [160].

3.4.2.2.1 Mn-TPPS

The water-soluble tetraphenylporphyrin derivative TPPS (Figure 3.8) has been most widely evaluated as a paramagnetic chelating ligand. Studies in tumour-bearing mice [192,193,194,195,196,197] have demonstrated increased uptake of the chelates into lymphomas, fibrosarcomas, colon carcinomas and breast cancer. In all cases, accumulation is seen almost immediately in the tumour; however, the best tumour : normal tissue ratios are achieved after approximately 48 hours when the complex has been excreted from normal tissues. This complex has also been used in imaging brain glioma [198]. Unlike Gd-DTPA, which perfuses out of the tumour, the porphyrin appears to bind tightly to the tumour cells resulting in enhancement of the tumour image for more than 4 days. The porphyrin complex is also highly efficient in distinguishing tumour from normal tissue and oedema. In this situation, it has been proposed that the selective uptake is through peripheral benzodiazepine receptors. These receptors are abundant in tumour cells but not in normal cells. The versatility of porphyrins suggests that there is potential, through chemical modification, of achieving selective uptake into almost any organ or tumour [198].

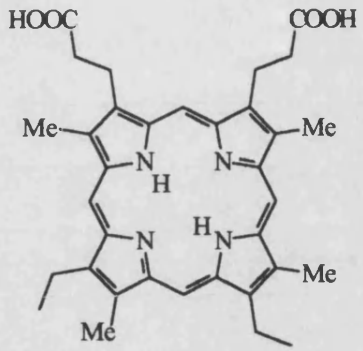



(76)

Figure 3.8

3.4.2.2.2 Mn-Mesoporphyrin, Mn-Protoporphyrin IX and Mn-Haematoporphyrin

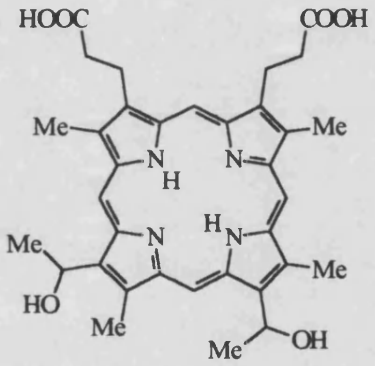
Although Mn(III)TPPS can be used successfully in many situations, contrast enhancement between the organ and tumour in the liver is not achieved. This is probably due to the rapid excretion of the complex through the kidney and urine [199]. Therefore, researchers have developed other synthetic porphyrins which are lipid soluble, in the hope that these will be excreted through the liver and bile and hence improve imaging of the liver. In a study comparing Mn-Mesoporphyrin (Figure 3.9) and Mn-TPPS, no enhancement of liver tissue image was seen with Mn-TPPS. With mesoporphyrin, there was significant enhancement of the signal intensity of normal liver parenchyma enabling contrast-enhanced images of both liver abscesses and metastatic disease within one hour. It was proposed, from radioactivity studies, that the mesoporphyrin derivative is taken up directly by the hepatocytes where accumulation occurs and is eventually excreted into the bile [199].




(77)

Figure 3.9 : Mesoporphyrin

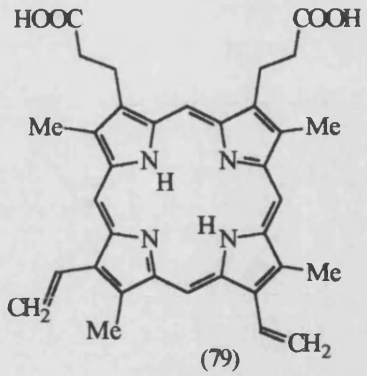
Similar results have been obtained with Mn(III) haematoporphyrin. However, this complex is retained for a long time in the liver and could give rise to toxic effects [199].




(78)

Figure 3.10 : Haematoporphyrin

Mn-Protoporphyrin IX is also targeted towards the liver in rat biodistribution studies. However, although it is a very stable complex, it has relatively low relaxivity and is not widely used [200].



(79)

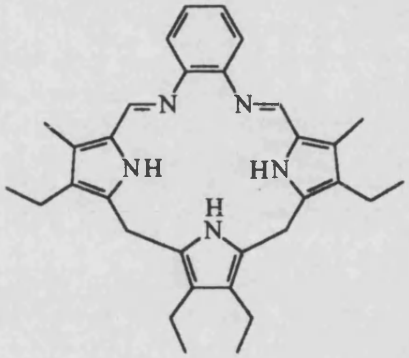
Figure 3.11 : Protoporphyrin IX

Mn-Mesoporphyrin has also been used in the imaging of rat glioma models. Increased enhancement of the tumour tissue image was achieved allowing the tumour to be distinguished from oedema and normal tissue. As with TPPS, the enhancement was prolonged in comparison to Gd-DTPA [184]. One added advantage of using lipid soluble porphyrins is their lack of photosensitisation. With Mn-TPPS, test animals experience both pigmentation of the skin and photosensitivity, with Mn-mesoporphyrin, these effects are not seen [199].

3.4.2.2.3 Gd-Porphyrins

As has been mentioned, the gadolinium ion is too large to form a stable complex with the porphyrin skeleton. However, as the relaxivity of $Gd^{3+}$ is higher there have been attempts to exploit the targeting properties of porphyrins to deliver Gd complexes. Recently, Hindré *et al.* [201] developed a conjugate of Gd-DTPA with a non-metallated tetraphenylporphyrin derivative. The relaxivity of this conjugate *in vitro* was greater than that of Gd-DTPA, probably owing to the increased size and hence increased correlation time. Enhanced images of human adenocarcinoma implanted into mice were achieved after 24 hours, demonstrating an increased tumour uptake of the Gd ions due to the tumour specificity of porphyrins. Similar results have been reported by Nakajima *et al.* [202].

An alternative approach is the use of modified porphyrins, known as texaphyrins, in which there is an expanded central region that contains five rather than four nitrogen atoms, allowing incorporation of Gd ions (Figure 3.12) [162].

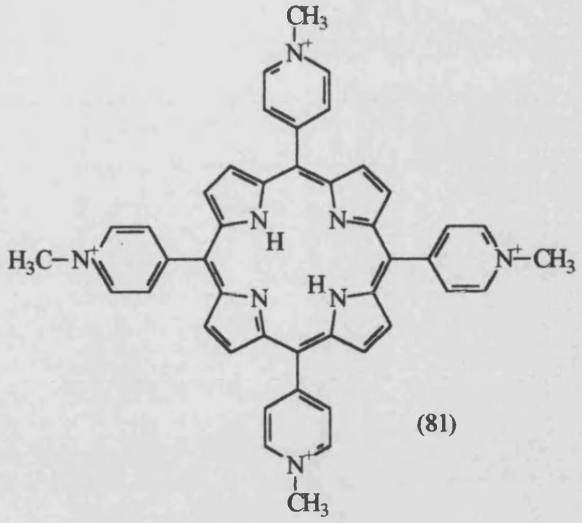



(80)

Figure 3.12

3.4.2.2.4 Indium Porphyrins

Recently a new class of metalated cationic porphyrins has been evaluated for targeting to malignant melanoma (Figure 3.13) [203]. Although these porphyrins contained radioactive $^{111}In$, the results provide information on the potential of targeting paramagnetic ions to one of the most diffuse metastatic cancers. These complexes gave excellent tumour localisation and tumour : blood ratios which were retained for over 72 hours. Interestingly, it was also possible to see where the porphyrin localised within the tumour. In this case, localisation was seen in the outer growing region whereas with TPPS distribution is mainly to the necrotic core.



(81)

Figure 3.13

3.5 Macromolecules

Proton relaxation enhancement can be achieved by the direct complexation of a paramagnetic ion to a macromolecule or through the attachment of a metal chelator to a macromolecule. The enhancement is due to an increase in the molar relaxivity of the complex. This effect is caused by the slow rotation of the paramagnetic ion which leads to an increased correlation time. The degree of effect depends upon the rigidity of the macromolecule and its tertiary structure. Relaxivities are higher when the paramagnetic ion is complexed with rigid globular protein molecules than with more flexible synthetic poly(amino acids). The effect of the increased correlation time is highest at higher field strengths where there is a large increase in relaxivity [160].

Both non-covalent and covalent complexes can be prepared. Non-covalent complexes, which result from a combination of electrostatic, van der Waals, hydrogen bonding and hydrophobic interactions, are more stable, through the action of many functional groups on the polymer, and exhibit higher relaxivities. In this situation, the metal is in equilibrium with metal in the bulk solution. On the other hand, although covalent attachments are attractive due to ease of characterisation, proteolytic

degradation within lysosomes can lead to release of the paramagnetic ion or chelator resulting in toxicity [160].

Complexation of a paramagnetic ion to a macromolecule has many advantages. Multiple paramagnetic ions can be attached to one large carrier molecule, therefore the molar dose of imaging agent can be reduced and hence toxicity can be reduced. More importantly, the macromolecular bound agents can be used to image the blood pool and hence situations where vascular integrity is lost such as in tumours and inflamed tissue [204].

### 3.5.1 Non-covalent complexes

Direct complexation of Gd and Mn to proteins such as concovalin A and bovine serum albumin result in enhanced molar relaxivities [160]. The synthetic polymer, poly(aspartic acid), has also been used to chelate $Gd^{3+}$ ions directly. Increased relaxivities were demonstrated with chelates bearing below 40 ions; above this level the relaxivity is reduced. This is probably due to interactions between the chelated ions [205]. Paramagnetic chelates can also be bound non-covalently to albumin. The hepatobiliary contrast agent Fe-EHPG has been successfully bound to albumin leading to a 2-3 times increase in relaxivity. The binding affinity can be enhanced by increasing the lipophilicity of the chelate [160].

### 3.5.2 Covalent Complexes

#### 3.5.2.1 Albumin

Considerably more interest has been directed towards the development of covalent macromolecular chelate complexes. Albumin is retained within the vascular space after intravascular injection. Conjugates of albumin Gd-DTPA have been used in studies in both rats and rabbits for imaging myocardial ischaemia [206,207]. In normal rats, [208,209] attachment of the chelate to albumin results in better imaging of the heart,

liver and brain at very low doses. Subcutaneous tissue and muscle are not enhanced, unlike with free Gd-DTPA. In this study, 16 chelates were attached to each albumin molecule and a threefold increase in relaxivity of the chelate was achieved on binding. Albumin is a natural polymer with high dispersity. It may be immunogenic and exhibits cardiovascular toxicity; thus other macromolecules have also been evaluated.

3.5.2.2. Dextran

Dextran, is available in a variety of sizes, has low toxicity and can be prepared to contain a biodegradable bond to ensure elimination of bound chelates. Chelation of Gd-DTPA to dextran leads to a large increase in relaxivity which is dependent on the number of chelates bound [210,211]. The chelates are successful in enhancing the imaging of the blood pool in rats, but are not as efficient as albumin chelates. This could be due to the larger molecular weight of albumin or to the higher permeability of the capillaries to dextran. Incorporation of a biodegradable bond enabled both superior imaging and excretion of the chelate in comparison to the free chelate [211]. Interestingly, with a large increase in the molecular weight of dextran, relaxivity is decreased. This is thought to be due to displacement of the bound water molecules, from the chelate, by neighbouring hydroxyl groups due to the polymer wrapping around itself [212].

3.5.2.3 Poly(Amino acids)

Synthetic poly(amino acids) have also been used for the formation of Gd-DTPA complexes. Poly(Lys)-Gd-DTPA allows enhanced images of the blood pool, pulmonary disease, such as oedema and embolisms, and the heart [209,213,214]. Mn (III) porphyrins have also been conjugated to poly(amino acids). Cross-linked polymers were produced through the coupling of protoporphyrin IX to a range of amino acids, poly(Glu) derivatised with lysine, poly(Lys), poly(Lys-co-Phe) and poly(Lys-co-Ala) [215]. All these conjugates exhibited higher relaxivities than the free porphyrin, this may be due to decreased porphyrin aggregation, as well as the increased rotational correlation time [216]. Highest binding of the porphyrin was achieved with the

poly(Glu)-based polymer, possibly owing to the incorporation of a long spacer unit. However, if the loading factor becomes too high, relaxivity enhancement may be lost due to interactions between the bound chelates. Attempts by these workers to produce a linear polymer, using a monofunctionalised porphyrin, resulted in an insoluble polymer [217].

3.5.2.4 Poly(ethylene glycol)

Poly(ethylene glycol) has also been used to change the relaxivity and toxicity of paramagnetic chelates. Desferrioxamine is an effective iron chelator but can lead to hypotensive effects on administration. A non-ionic PEG derivative has been prepared which was non toxic and allowed enhanced imaging of the kidney and liver in dogs [218].

More recently, Gd-DTPA has been attached to PEG. With polymers of MW above 20,000 Da increased contrast enhancement of the blood pool and tumours was observed [219].

3.5.2.5 Dendrimers

Recently it has been suggested that dendrimers or cascade polymers would be more effective relaxation enhancers, owing to their high rigidity and potential for binding a large number of ions. The first branched polymers used were based on polyethyleneimmine, which can directly chelate paramagnetic ions [220]. However, dendrimers based on Starburst ® have also been evaluated. Monodispersed chelates could be produced with varying numbers of chelating ligands, depending on the cycle of polymerisation, allowing between 11 and 170 Gd ions to be bound to the DTPA-dendrimer. The relaxivity of the macromolecule with 170 Gd ions bound was equivalent to that produced by 1074 free Gd-DTPA molecules and was over ten times greater than that achieved with linear polymers. Interestingly, no mass effect is seen with these macromolecules; increasing the mass does not lead to a decrease in relaxivity [212]. The wide variety of molecular sizes available with this system leads to a

diverse set of applications, small complexes can be used for extravascular imaging of tumours and brain, whereas large complexes can be used for the imaging of vasculature.

### 3.6 Particulates

Imaging of small lesions in liver and spleen is difficult to achieve using magnetic resonance imaging. Although images can be enhanced using Gd-DTPA, the enhancement is transitory due to the fast blood and urinary clearance of the chelate and the lack of an active uptake mechanism [221]. This can be overcome by the use of higher doses; however, higher and potentially unacceptable toxicity will ensue.

#### 3.6.1 Liposomes

An alternative approach is to use either particles or liposomes containing paramagnetic ions or chelates. Liposomes, due to their size, are retained in vasculature for a prolonged time period and are removed from the circulation either within the lung or by the action of phagocytic cells of the reticulo-endothelial system. In the normal liver, liposomes can be taken up by the Kupffer cells or, if very small, can be taken up by hepatocytes. Hepatic tumours lack phagocytic cells and therefore cannot take up liposomes [222]. Therefore, by preparing liposomes containing contrast enhancement agents, the normal tissue image can be enhanced relative to that of the tumour and a negative image of the tumour can be obtained.

There are three approaches to the incorporation of contrast enhancement agents into liposomes. Firstly, a water soluble chelate, such as Gd-DTPA, can be incorporated into the aqueous core of the liposome. Secondly, a lipid-soluble chelate can be incorporated into the pre-formed lipid bilayer. Finally, the chelate can be modified to prepare ampipathic derivatives which can be used, along with other lipids to form the bilayer [222].

3.6.1.1 Chelates in Aqueous Core

Liposomes have been prepared in which Gd-DTPA is enclosed within the aqueous core [223,224,225]. These liposomes have been tested for their relaxivity *in vitro*, and stability in serum and saline. Liposomes were prepared in a variety of sizes between 50 and 400nm in diameter from egg phosphatidyl choline (EPC) [224]. The relaxivity of the paramagnetic ion in liposomes was less than for free paramagnetic chelates and was highest in the smallest liposomes, which have the largest surface area : volume ratio. It has been postulated that relaxivity of a chelate-containing liposome is directly proportional to the flux of water molecules across the liposomal membrane which is itself related to the surface area : volume ratio. The loss of image intensity due to decreased relaxivity can be overcome if the agent becomes concentrated in the desired region. With small Gd-DTPA liposomes there is increased uptake into the liver, vascular space and bone marrow and the body clearance is increased to 3.5 days in mice. This distribution allows imaging of small hepatic metastases with dose as low as 0.025 mmol/kg of liposome [224].

Acute and sub-acute toxicity tests have also been performed. The acute toxicity of the liposomal preparation is not dissimilar to that of standard chelates. However, on continued administration, the mice treated with the liposomes demonstrated both enlarged livers and spleens. It has been suggested that this effect may not be seen if liposomes containing Gd-DOTA, where the ion is bound more tightly, are used [224].

3.6.1.2 Chelates in Lipid Bilayer

The main disadvantage with entrapping the chelate within the aqueous core is the reduced relaxivity. If the paramagnetic ion could be presented to the desired cells on the surface of liposomes this could be overcome. Thus, a second type of liposome has been prepared in which the chelating ligand is derivatised to be lipid-like, allowing it to be incorporated into the lipid bilayer [226]. The amphipathic chelate Gd-DTPA-SA was prepared and mixed with EPC and cholesterol to form liposomes. Maximal relaxivity was seen with a 33.3% incorporation of the modified ligand; in studies *in*

*vitro,* the relaxivity of spleen and liver was increased by 110% and 66% respectively. Biodistribution was also evaluated, the liposomes rapidly clearing from the blood and accumulating in liver and spleen from which they are cleared after 11 days. No toxicity was seen. Following on from this work [227], a second ampipathic ligand has been prepared which would allow more rapid clearance from the liver. Thus the ligand was bound to the lipid *via* a stearyl ester (SE) group rather than through a stearyl amide (SA) group. With a 33.3% incorporation comparable $T_1$ signal enhancement was observed as with Gd-DTPA-SA. However although the liposomes clear as rapidly from the blood and accumulate in the liver, after 2 days 50% of the liposomes are cleared. In a further study [228], liposomes were prepared containing the derivatised ligand DTPA-SA, in a variety of concentrations. $Gd^{3+}$, $Mn^{2+}$ and $Fe^{3+}$ were then incorporated into the chelate and the liposomes were evaluated. The $Fe^{3+}$ liposomes were unstable on formation and could not be evaluated. The $Mn^{2+}$ liposomes were unstable in serum; however, they accumulated rapidly in the liver and were excreted rapidly, allowing good quality images to be obtained. The $Gd^{3+}$ liposomes, in contrast, were stable in plasma, were accumulated rapidly into the liver and were retained, allowing images to be obtained even after 24 hours.

Thus, liposomes are highly attractive for specific delivery to the liver and spleen. Incorporation of the ligand in either part of the liposome allows enhanced images with lower doses of paramagnetic ions. It has been suggested that this technology could be further exploited. Liposomes can be diverted from the reticulo-endothelial system by using large doses or very small vesicles or by 'blocking' the phagocytic cells by pre-treatment with empty liposomes. These alterations would allow blood pool imaging. Alternatively, targeting moieties could be incorporated into the lipid bilayer to allow cell specific targeting [162].

### 3.6.2 Particles

It has also been suggested that particles can be used for the carrying of chelates. In a study of cross-linked starch microspheres incorporating DTPA, a variety of paramagnetic ions were coupled and exhibited increased relaxivity over simple

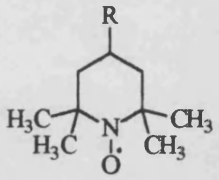
chelates. The increase in relaxivity is possibly due to the swelling of the microsphere in water, allowing passage of water protons close to the paramagnetic ions [229]. Microspheres offer potential as contrast enhancement agent carriers and deserve further evaluation.

## 3.7 Molecular Oxygen and Nitrogen Stable Free Radicals

Other paramagnetic species, apart from metallic ions, have been evaluated as contrast agents [162].

Molecular triplet oxygen has been used in MRI of the lung through inhalation. In these studies, signal enhancement is seen in the left oxygenated lung in comparison to the right lung. However, this alteration is insufficient to warrant further investigation [230].

A more promising approach, utilising paramagnetic molecules, is the use of stable free radicals or 'spin labels', particularly nitroxyl stable free radicals. Nitroxyls are labile to redox reactions; steric protection confers stability. Thus the moiety is relatively unreactive in most conditions and remains stable even when heated above 100°C and under a wide range of pH conditions [230].




(82)

Figure 3.14 : Piperidine Nitroxyl Stable Free Radical

These compounds provide strong relaxation enhancement because of electron paramagnetism that is comparable to $Cu^{2+}$ or $Fe^{3+}$ paramagnetic ions. They produce decreases in both $T_1$ and $T_2$. However, these compounds offer one major advantage

over other paramagnetics. They can be chemically modified through the R group or attached to targeting molecules or biomolecules to allow selectivity of action. Previously, these compounds have been successfully attached to drugs such as propranolol and steroids to study drug metabolism [230].

Nitrogen stable free radicals have mainly been used as spin probes to study biochemical systems using electron spin resonance spectroscopy. These studies demonstrate that the compounds persist for a prolonged period in biological tissues without electron pairing or reduction taking place by the formation of hydroxylamines [231]. They have been mainly used in MRI to asses renal function and identify renal abnormalities. In an early study [231], the compound TES (N-succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) was used to image the kidney and asses renal function in animals with renal ischaemia, congestion or atrophy. The free radical is rapidly excreted in urine with a clearance rate equal to the glomerular filtration rate. This allows a transient increase in signal intensity in the kidney thus enabling increased determination of renal abnormalities.

Nitrogen stable free radicals have not been widely used in MRI, as little advantage is obtained over simple paramagnetic chelates. However, one interesting use of nitrogen stable free radicals is in the imaging of hypoxic tumours. These compounds can be used as probes of oxygen concentration, as in low oxygen reducing environments such as in hypoxia, the compound is reduced and the signal enhancement is lost. [230]

## 3.8 Superparamagnetic particles

Small ferrite particles have superparamagnetic properties, that is, they are easily magnetised to a high magnetic moment but the magnetism is lost once the external magnetic field is removed [164]. An example of a ferrite particle is magnetite, a natural compound $Fe_3O_4$, found in many living organisms, which is often found in combination with maghaematite and haematite $Fe_2O_3$ [164]. Superparamagnetic particles exert effects on both $T_1$ and $T_2$, but the effect on $T_2$ outweighs all other effects leading to a loss of signal in the affected organs [162]. Magnetite particles are

administered as polymer coated particles to allow the formation of aqueous solutions, and alter particle size. Although both starch and albumin have been used, the majority of studies have been performed on AMI-25, a commercial product, in which the iron oxide is coated in dextran [162].

### 3.8.1 Dextran-Coated Particles

Initially, studies were carried out using relatively large particles designed to be captured by the cells of the reticulo-endothelial system allowing imaging of the liver and spleen [232,233]. AMI-25 has been used in clinical trials for the diagnosis of liver cancer [234,235], cerebral vasculature [236] and cardiovascular imaging [237]. Studies [235] in 15 patients with known liver cancers demonstrated hyperintense imaging of these lesions. Normal liver cells, *i.e.* Kupffer cells, take up the particles by phagocytosis and there is a loss of signal in these cells and the surrounding hepatocytes giving a negative image of the liver. Abnormal cells loose the ability to phagocytose, so do not take up the contrast agent and thus appear hyperintense on the image. Although 90% of the particles were cleared by the liver in 1 hour, some remain circulating allowing imaging of other systems, particularly the heart [237] and brain [238]. Clearance from the liver is demonstrated in humans within 24 hours of treatment [235]. The breakdown of the particle does not lead to abnormalities or toxic effects as iron is a major constituent of the body.

### 3.8.2 Albumin Microspheres

Studies with microspheres, in which magnetite is imbedded in an albumin matrix, have also demonstrated enhanced imaging of hepatic tumour models in rabbits. These are slightly larger particles (1-5 μm) but they are still efficiently taken up by the reticulo endothelial system and are rapidly cleared from the blood stream. There is long term retention of these microspheres. However, no toxicity or immunogenicity has been demonstrated; after 2 months there is no evidence of hepatocellular damage or fibrosis [239].

### 3.8.3 Ultrasmall Dextran Particles

Ultrasmall dextran-magnetite particles have also been developed by particle-size sorting of AMI-25. In normal rats, [240] over 24 hours, accumulation was higher in the lymph nodes, bone marrow, liver and spleen and the blood half-life was increased from 6 minutes for AMI-25 to 81 minutes. These particles are small enough to transmigrate through capillaries and thus are taken up by the lymphatics. Without contrast enhancement, there is a considerable overlap in the relaxivities of both normal and metastatic lymph tissue. However, on administration of ultrasmall particles, metastatic lymph tissue is visualised as hyperintense regions, in comparison to normal tissue, as it cannot phagocytose [241].

With these developments, have come further approaches to targeting, away from the reticulo-endothelial system. One approach is the ferrosome, this is a small vesicle that contains superparamagnetic particles in the central aqueous compartment. These have been evaluated in the imaging of lymph nodes, pulmonary emboli, adenocarcinomas and hepatomas. In tumour tissue, they are taken up by the macrophages located at the surface of the tumour giving a rim of low signal intensity. Alternatively, the superparamagnetic particles can be coated in polymers with intrinsic targeting properties. Particles coated with arabinogalactose can be targeted to hepatocytes *via* the asialoglycoprotein. This achieves imaging of the liver with reduced doses in comparison to targeting to Kupffer cells [162].

## 3.9 Conclusion

Many contrast enhancement agents have been developed for MRI. The majority are based on the relaxation properties of paramagnetic ions. Complexation or conjugation, of paramagnetic ions or small chelates of paramagnetic ions, to macromolecules results in an enhanced relaxivity. Thus images with greater contrast can be produced. This coupled with the passive targeting of macromolecules to tumour tissues enables accurate well-contrasted images of tumours.

# CHAPTER FOUR

## SYNTHETIC STRATEGIES

As can be seen from the preceding chapters, the concept of macromolecular pro-drugs has been widely investigated, particularly in the treatment of cancer. The technology is also suitable for the preparation of macromolecular contrast enhancement agents. These are particularly interesting; binding of the agent to a macromolecule not only increases the relaxivity of the conjugate and, hence, the contrast enhancement but also allows accumulation in the tumour to be imaged, due to the EPR effect.

In this thesis, work is presented on the development of a new series of polymers suitable both for use in macromolecular pro-drugs and macromolecular contrast agents.

The main disadvantage with previously used synthetic polymers, such as HPMA, has been their lack of degradability *in vivo*. This limits their use in pro-drugs to conjugates that have a molecular weight lower than the renal filtration threshold, *i.e.* to conjugates that can be excreted. This may, however, reduce the effectiveness of the conjugates by limiting the extent of accumulation of the macromolecule in a tumour, a size dependent process.

Thus, an ideal polymer would combine biodegradability of the backbone, to allow excretion, with the advantageous properties of synthetic polymers, such as water solubility, reduced immunogenicity, and low toxicity. An approach to this is to prepare a co-polymer of a synthetic polymer with a biodegradable unit. From the work of Duncan, Kopecek and Trouet, it is apparent that some small peptide sequences can show selective biodegradability *in vivo*. That is, they are degradable in lysosomes but not in the blood stream. If a polymer can be prepared incorporating such a sequence, it can be used as a high molecular weight pro-drug without fear of unwanted accumulation. High tumour tropism will be achieved; however, after

delivery of the drug, through the endocytic pathway, the degradable unit would be cleaved in the lysosome to yield smaller oligomers which can be excreted.

To this end, this thesis examines the preparation of a co-polymer of poly(ethylene glycol) and a degradable unit for use in macromolecular conjugates. Ulbrich *et al* [78] have prepared a number of degradable co-polymers of PEG in which the degradability is imparted by just one amino acid, Phe, and Yokoyama [153,154], a degradable co-polymer of PEG and poly(aspartic acid). However, these polymers do not display selective biodegradability; thus it was proposed to incorporate the well-known sequence GlyPheLeuGly. This has proved selectively degradable in two situations; as a crosslink in HPMA and to link a drug to HPMA, however as yet it had not been evaluated as a in chain sequence.

However, as both PEG and the peptide unit have no side-chain functionality, a further unit must be incorporated into the co-polymer to allow attachment of a drug molecule. This can be achieved in many fashions; however, the peptide unit can be easily functionalised by the inclusion of a further amino acid to the peptide sequence. Many amino acids are suitable. Glutamic acid and lysine have been evaluated allowing the development of a number of coupling reactions.

Polymerisation of PEG and the peptide unit can be achieved in many ways. PEG can be modified to an α,ω-bis-nucleophile, such as the α,ω-di-amine, or to an α,ω-bis-electrophile, such as the α,ω-diglycidyl ether. The peptide unit can then be suitably prepared to provide suitable reactive termini for polymerisation. In this work, the synthetic strategy employed for the polymerisation is the reaction between an α,ω-diglycidyl ether derivative of PEG and α,ω-bis(secondary amino) peptide unit. The secondary amine is essential to avoid cross-linking or branching of the polymer. A secondary amine can be easily incorporated at the N-terminus of the peptide unit by extending the peptide chain further by inclusion of the amino acid sarcosine (N-methylglycine). To provide a secondary amine of similar reactivity at the C-terminus is more complex. A retro-inverso unit must be incorporated to change the sense of the

peptide chain. This reveals an amine at the C-terminus which can be coupled with sarcosine to give the α,ω-bis(secondary amine).

For preliminary evaluations, a conjugate suitable for evaluation as a macromolecular contrast agent was proposed. Unlike a drug molecule, which often has a number of functional groups, thus requiring protecting group strategies, contrast enhancement agents can be prepared which are monofunctional. To this end monofunctional derivatives of tetraphenylporphyrin were chosen for attachment to the ε-amine of lysine or the γ-carboxylic acid of glutamic acid in the peptide unit. This can be used as a chelator of manganese (III) and similar derivatives have been previously used as contrast enhancers. To ensure reliable loading of the porphyrin onto the polymer, it was proposed to prepare peptide-porphyrin conjugates which could then be polymerised with PEG. Metal incorporation, however, would be a final step, due to difficulty of NMR analysis of metal-bearing compounds.

Drugs are often attached to macromolecules *via* spacer units. On the whole these are degradable to allow release of the drug *in vivo* and hence activity. With macromolecular contrast enhancement agents, however, the chelator is more effective when coupled to the macromolecule. Thus, in this work, a non-degradable spacer was incorporated between the porphyrin and peptide in a number of monomers.

Thus, the preparation of a monofunctionalised porphyrin suitable for attachment to both glutamic acid and lysine is presented in this thesis. The preparation of a number of potentially degradable peptide units is discussed; attachment of the porphyrin to the peptide monomers is also reported. Preliminary experiments on the polymerisation reaction and metallation are also discussed.

# CHAPTER FIVE

# PORPHYRIN SYNTHESIS

## 5.1 Introduction

In order to prepare a linear polymer bearing a pendant porphyrin moiety, it was essential to synthesise a monofunctionalised porphyrin derivative. Most natural porphyrins, for example protoporphyrin IX, contain many functional groups suitable for attachment to polymers. However, because they are multi-functionalised, cross linked polymers are liable to be formed. Thus, a monofunctionalized synthetic porphyrin was required. The most simple monofunctional porphyrins are those with a *meso* alkyl substituent. These, however, are difficult to synthesise in high yields [244]. *Meso*-tetraphenyl porphyrin (TPP) and its monofunctionalised derivatives are more easily synthesised and, as this type of porphyrin are known to localise in tumour cells [193], our work has been focused on the development of TPP derivatives.

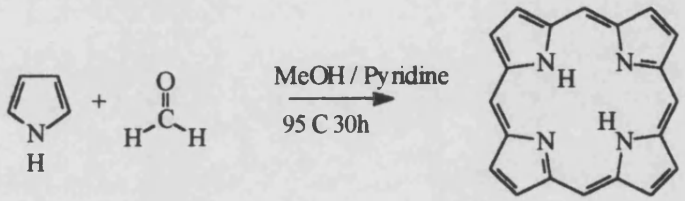
The porphyrin is to be coupled to amino acids in the peptide sequence. Thus, it must be suitably functionalised to allow coupling to either amino or carboxylic acid side chains of amino acids. Therefore, either an carboxylic acid or amino derivative of TPP is required. As the porphyrin is being used as a model of a drug, it should be attached to the peptide *via* a spacer, although this does not need to be degradable in this situation.

## 5.2 Synthesis of Tetraphenylporphyrin

The unsubstituted porphine ring, the basis of all porphyrins, is difficult to synthesise. In addition to the desired product, chlorin rings can be formed. Although these can be easily oxidised to the porphine, yields of the porphine are still low. The main approach to the synthesis of porphyrins is *via* a condensation reaction between pyrrole and an

aldehyde. In the case of porphine, this aldehyde is formaldehyde; for TPP, benzaldehyde.

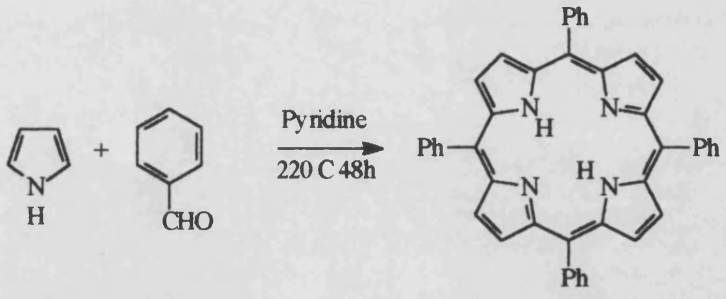
Rothemund [242] reported the first synthesis of porphine **(85)**. In this method, condensation was achieved by heating the reactants in a methanol / pyridine mixture. This was performed in sealed tubes and thus was an anaerobic reaction. (Scheme 5.1) [242].




(83) (84) (85)

Scheme 5.1

Using these conditions for the preparation of TPP, resulted in the formation of a large amount of chlorin [243]. Modified reaction conditions, heating at 220°C in pyridine, allowed enhanced preparation of TPP. However, even using this method, the yield of porphyrin was less than 5% [243] (Scheme 5.2). Addition of zinc acetate to the reaction mixture, as a template, resulted in slightly enhanced yields, 10% as the zinc porphyrin [244].

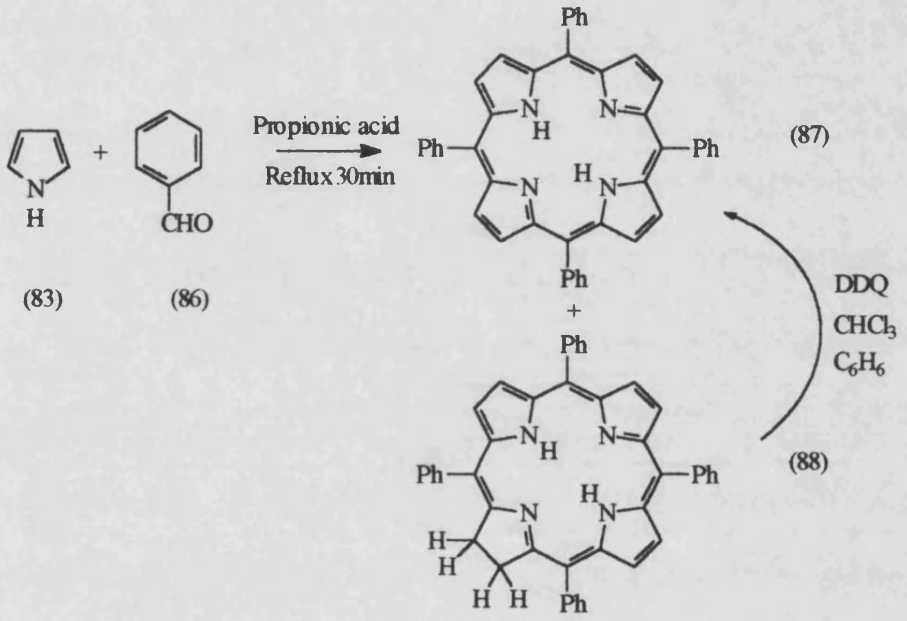



(83) (86) (87)

Scheme 5.2

In 1967, Adler *et al* [245] presented an improved synthesis of TPP. In this method, pyrrole and benzaldehyde were heated for 30 minutes in refluxing propanoic acid. On

cooling, TPP crystallised from the solution and could be separated by filtration. Using this approach, consistent yields of 20% could be achieved. Even higher yields, 35-40%, could be achieved using acetic acid as the solvent [246]. However the product does not crystallise from this solvent and thus is considerably more difficult to purify. Some of the corresponding chlorin is also formed which can be easily removed by chromatography or sublimation [245]. Alternatively it can be converted to the porphyrin by oxidation of the crude material with 2,3 dichloro-5,6-dicyanobenzoquinone (DDQ). (Scheme 5.3) [244,246].




Scheme 5.3

Numerous derivatives of TPP have been prepared by both the Adler and Rothemund approaches, including the tetrakis *para* chloro, *para* methoxy, *para* methyl and *ortho* and *para* nitro derivatives [244,246,247].

## 5.3 Synthesis of mono-functionalised derivatives of TPP

There are several approaches to the preparation of mono-functionalised porphyrins. Buchler *et al* [248] prepared mono-substituted porphyrin derivatives using a Vilsmeier formylation at a pyrrole β position.. More recently, Dolphin *et al* [249] have developed a

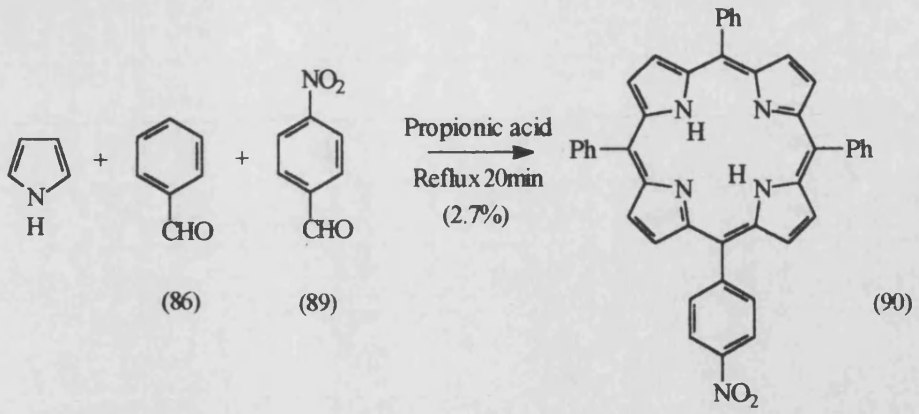
mono functionalised porphyrin with an iodinated *meso*-position. This was suitable for the attachment of alkynes, including 17 α-ethynyltestosterone, *via* a Heck coupling.

The majority of mono-substituted porphyrins prepared have been based around substituents on *meso*-phenyl groups. Functionalised TPP can be prepared using two main methods, both based on condensation reactions, by modification of the Adler-Rothemund procedure or by the methods of Lindsey *et al.*

Both methods are based on a mixed condensation procedure. Functionality is incorporated into the macrocycle in the form of a modified benzaldehyde. A mixture of the functionalised benzaldehyde and benzaldehyde is treated with pyrrole. Thus, the ratios of aldehydes will determine the amount of mono functionalised porphyrin prepared. Using a 1 : 3 functionalised to unfunctionalised ratio, five main products will be formed. The majority product (42.2%) will theoretically be the mono substituted porphyrin. However, large quantities of both underivatized (31.6%) and disubstituted compound (21.1%) will also be prepared along with tri and tetrakis substituted derivatives. In the majority of cases, it is possible to separate the desired product from the side products by column chromatography with some difficulty.

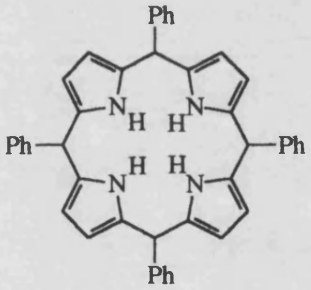
### 5.3.1 Mono amino derivatives

In order to obtain the mono-amino derivative, the mono-nitro compound is first prepared and then reduced. Hasegawa [250] prepared the mono-nitro derivative, using a modification of the Adler-Rothemund procedure. Using this approach only an unacceptably low yield (2.7%) was achieved (Scheme 5.4).




Scheme 5.4

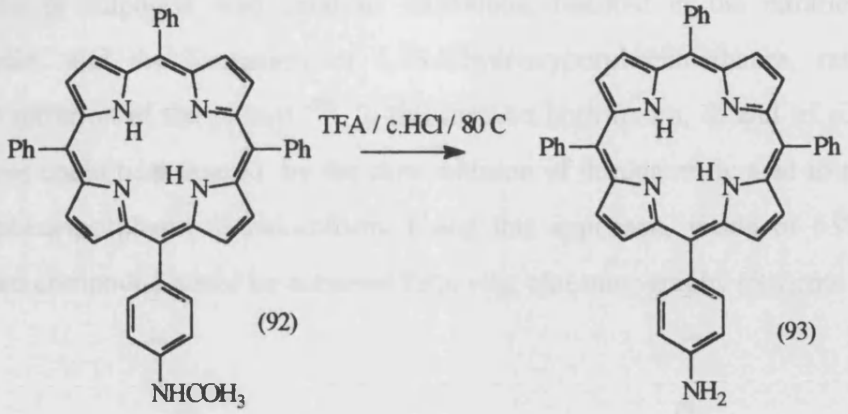
Lindsey *et al* have developed gentler conditions, in which condensation takes place at room temperature, for the preparation of monofunctionalised porphyrins. In one approach, the aldehydes and pyrrole in chloroform are treated with $BF_3(OEt_2)$ in ethanol in the absence of air. This co-catalysis results in the formation of a porphyrinogen skeleton (Figure 5.1).




(91)

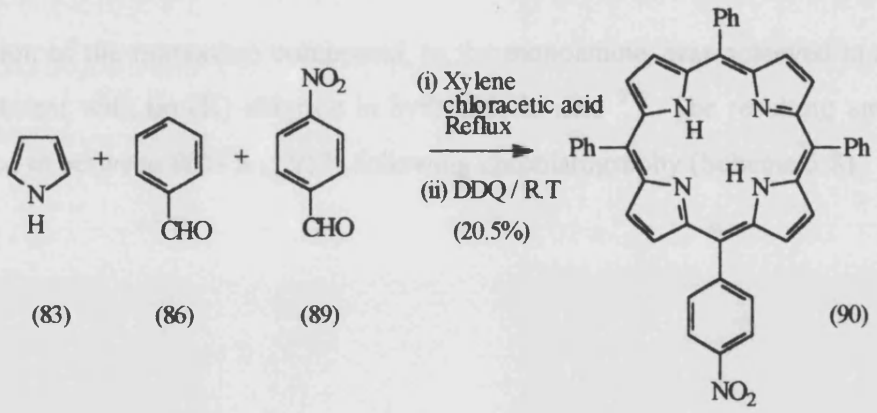
Figure 5.1

This is then oxidised, by treatment with DDQ and $O_2$, to the desired porphyrin. In a slightly modified approach, condensation is initiated by the addition of trifluroacetic acid, and oxidation is achieved by treatment with *p*-chloranil [251,252,253]. In an alternative approach Lindsey *et al* have prepared monoamino porphyrin **(93)** from the acetamidophenyl derivative [254] (Scheme 5.5).




Scheme 5.5

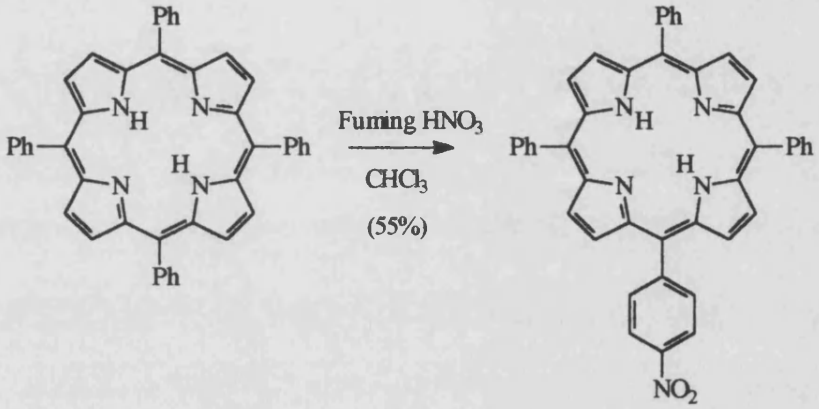
Evstegneeva *et al* [255,256] developed a modification of the Lindsey methods in the formation of mono nitro porphyrin. In this approach, the reactants are heated at reflux in a mixture of xylene and chloroacetic acid for one hour. After cooling, partial oxidation is achieved by the addition of DDQ. The resulting crude material is then applied to an alumina column, and eluted with xylene for 10 days. Whilst the product is on the column, further oxidation takes place to give the porphyrin. These workers found difficulty in separating the mono functionalised porphyrin from tetraphenyl porphyrin, achieving a crude yield of 21 %. (Scheme 5.6)




Scheme 5.6

A completely different method for the preparation of mononitro TPP was reported by Kruper *et al* in 1989 [257]. This involves direct functionalisation of TPP by fuming nitric acid. Previous attempts to prepare the mononitro compound, using either radical

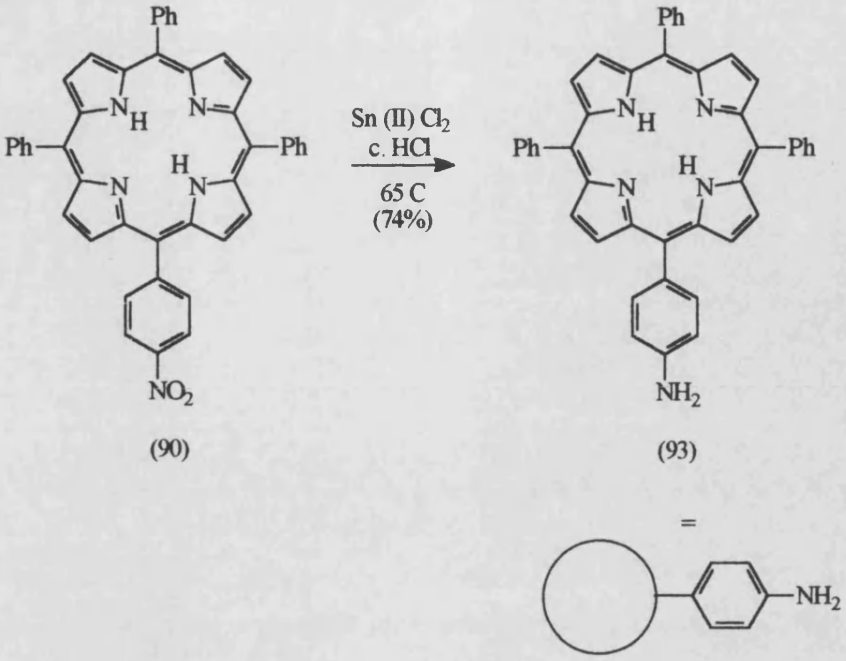
conditions or sulphuric acid catalysis conditions, resulted in the nitration of the macrocycle, and the formation of 5,15-dihydroxyporphodimethanes, rather than selective nitration of the phenyl [258]. In this method both mono, di and tri substituted derivatives could be prepared, by the slow addition of fuming nitric acid to a solution of tetraphenylporphyrin in chloroform. Using this approach, yields of 55% of the mononitro compound could be achieved following chromatography (Scheme 5.7).




(87) (90)

Scheme 5.7

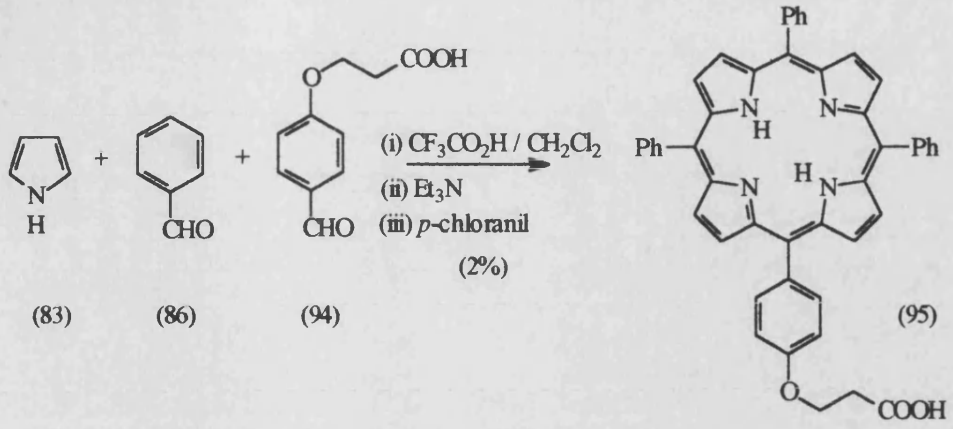
Reduction of the mononitro compound, to the monoamine, was achieved in all cases by treatment with tin (II) chloride in hydrochloric acid [250]. The resulting amine was prepared in between 80% and 95% following chromatography (Scheme 5.8).




Scheme 5.8

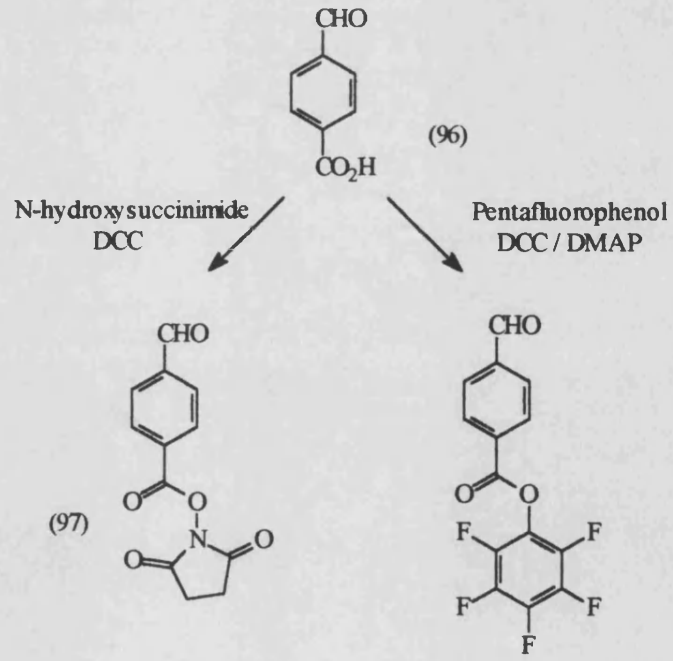
5.3.2 Mono carboxylic acid derivatives

Carboxylic acid derivatives of porphyrins have been prepared in low yields by both Adler-Rothemund and Lindsey methods [247]. Lawley and Threadgill prepared a mono carboxyethoxy derivative of tetraphenylporphyrin using Lindsey conditions (Scheme 5.9). On a small scale, a yield of 2.2% was achieved. However, attempts to scale up this reaction resulted in a mixture of products which could not be separated [259].




Scheme 5.9

In order to couple a carboxylic acid derivative of tetraphenylporphyrin to a peptide, it must first be activated. Lindsey *et al* [252] have prepared a series of aldehydes, in which the carboxylic acid function is both activated and protected, by derivatisation to the N-hydroxysuccinimide or pentafluorophenyl ester. These aldehydes are compatible with the conditions for mixed aldehyde condensation allowing the formation, in one step, of a derivatised and activated porphyrin (Scheme 5.10).




Scheme 5.10

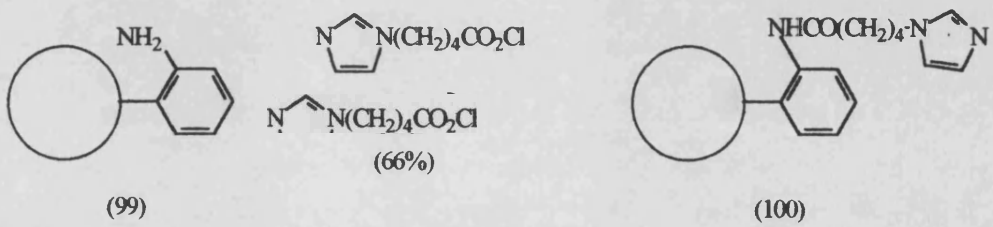
However, in this project, it was decided to prepare (4-aminophenyl)triphenylporphyrin (monoaminoporphyrin). This could be further derivatised, by incorporation of a spacer, to provide a carboxylic acid function or an extended amino derivative. Therefore, it would be possible to achieve coupling both to lysine residues and glutamic acid residues in the peptide.

Tetraphenylporphyrin was prepared using the Adler method on a large scale. Consistent yields (19%) of crude TPP were achieved using this method. The crude crystals formed on cooling were not purified further.

Mononitration was achieved using the method of Kruper *et al* [257]. Chromatography enabled the isolation of both (4-nitrophenyl)triphenylporphyrin (mononitroporphyrin) and a di nitro derivative. The latter was formed in very low yields. The mononitro compound was successfully reduced (91%) on treatment with tin (II) chloride in concentrated hydrochloric acid to give the desired amino porphyrin. For both reactions, increases in scale resulted in decreased percentage yields due to difficulties in chromatographic isolation.

## 5.4 Spacer Incorporation

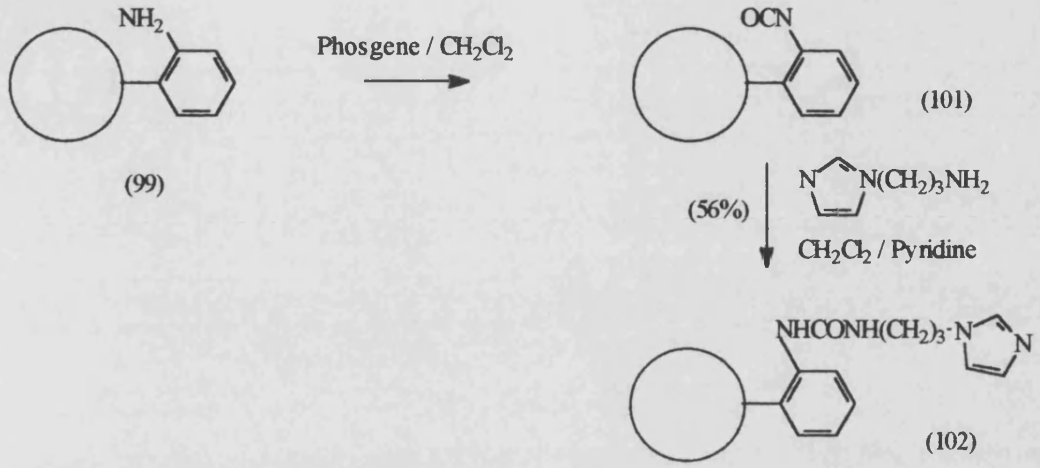
Many methods have been developed to extend the functionalisation of porphyrins. Collman *et al* [260] developed two techniques for the attachment of aliphatic spacers to the *ortho*-amino-tetraphenylporphyrin. They found that standard approaches, using peptide chemistry techniques, such as direct DCC and active ester couplings, did not result in conjugate formation. Acyl chloride derivatives of the spacers can however be easily coupled to aminoporphyrin to provide an amide linkage (Scheme 5.11).




(99) (100)

Scheme 5.11

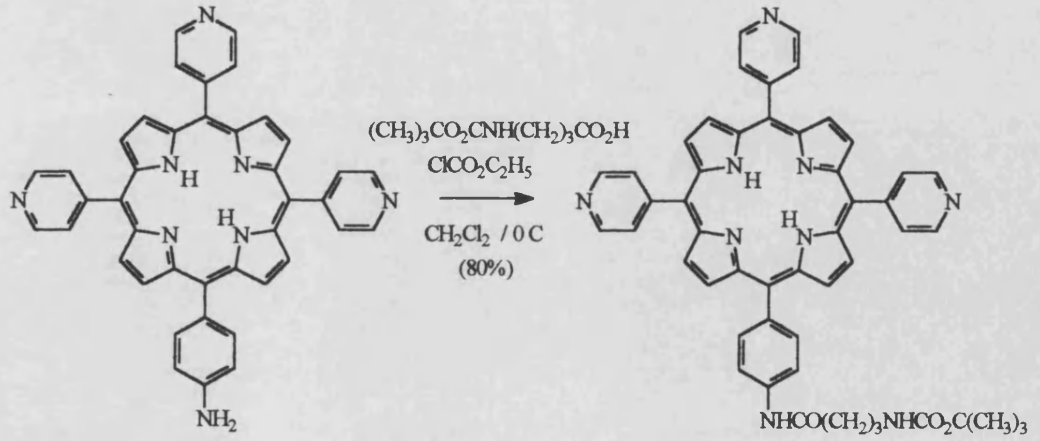
In an alternative approach, the spacer can be incorporated through urea linkages [260]. The porphyrin amine can be activated, by phosgene, to the isocyanate, thus allowing easy coupling with amine spacers (Scheme 5.12).




Scheme 5.12

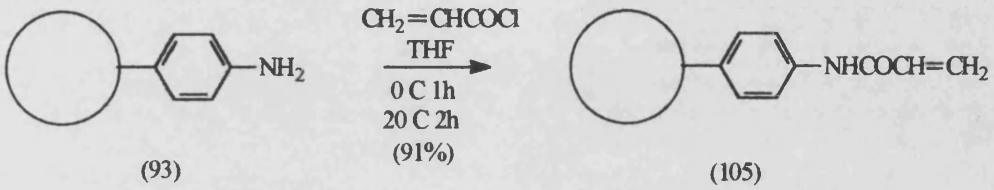
Hunter and Sarson have also used acid chloride coupling procedures to provide a spacer between a porphyrin and pyridine [261]

Aminomethylene chains can also be attached to a monoaminoporphyrin using a mixed anhydride method. Ding *et al* [262] prepared a mixed anhydride of Boc-aminobutanoic acid with ethyl chloroformate. Coupling to the porphyrin amine was achieved in 80% yield (Scheme 5.13). Deprotection afforded an extended amino porphyrin derivative.




(103) Scheme 5.13 (104)

An interesting modification of monoamino tetraphenylporphyrin was developed by Hasegawa *et al* [250,263]. In this approach, acrylic or methacrylic acid chlorides were coupled with the porphyrin to form vinyl derivatives (Scheme 5.14). These derivatives were then polymerised, via a radical co-polymerisation, with styrene to give a number of porphyrin-containing polymers. Although, these polymers were developed as artificial oxygen carriers, they could be evaluated as relaxation enhancers in MRI.



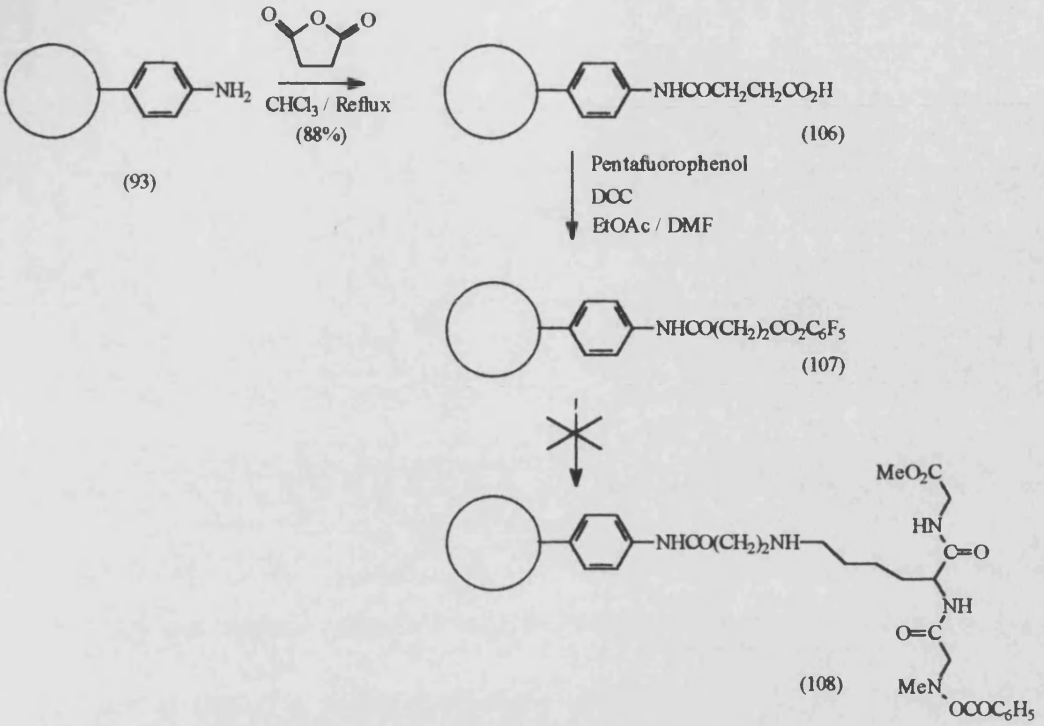

(93) (105)

Scheme 5.14

## 5.5 Synthesis of Spacer Derivatives

It was intended to incorporate an aliphatic spacer, of between 2 and 5 methylene units, onto the porphyrin. Initially, the aminoporphyrin was treated with succinic anhydride, to provide a carboxylic acid derivative. This, following activation by standard peptide method was expected to be coupled to the ε-amino group of lysine residues in the peptide.

The aminoporphyrin was heated with an equivalent of succinic anhydride in chloroform; 4-oxo-4-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)-butanoic acid precipitated in 87% yield (Scheme 5.15).

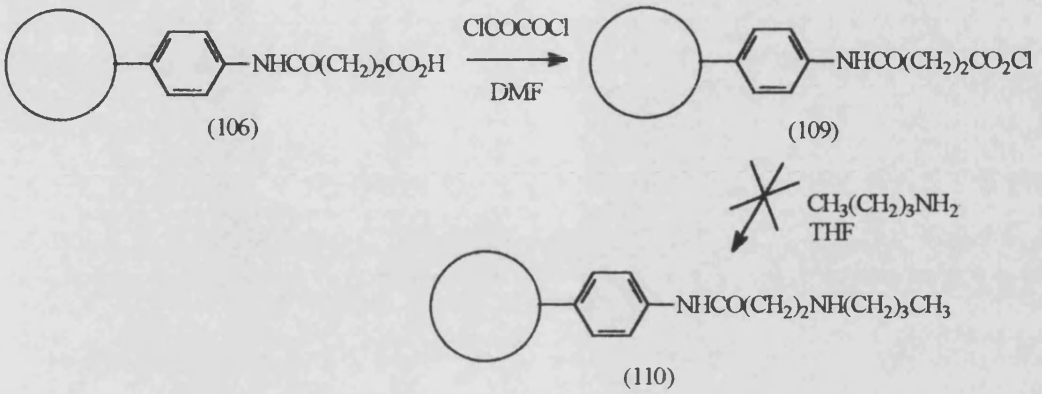



Scheme 5.15

This was treated with pentafluorophenol and DCC in an EtOAc / DMF mixture to prepare the activated pentafluorophenyl ester of the porphyrin. A product, believed to be the desired ester, formed slowly. After two days, the solvents were evaporated and the crude material was added to a peptide sequence with a deprotected ε-amino group. No coupling was observed on both stirring at room temperature or on heating at 40°C for two days. Addition of DMAP, followed by heating at 60°C resulted in the formation of two new porphyrin containing compounds. Chromatography allowed isolation of these products; however, these could not be identified. This may be due to a lack of purity of the peptide rather than difficulties with the coupling procedure. However, the difficulties encountered in the formation of the active ester led to the investigation of other activation procedures.

Activation of the carboxylic acid as the acyl chloride was then attempted. 4-Oxo-4-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)butanoic acid in THF was

treated with oxalyl chloride. After one hour, total conversion of starting material was observed by TLC. Following the removal of excess reagent, the acid chloride was treated with butylamine, a model for the lysine ε-group (Scheme 5.16).

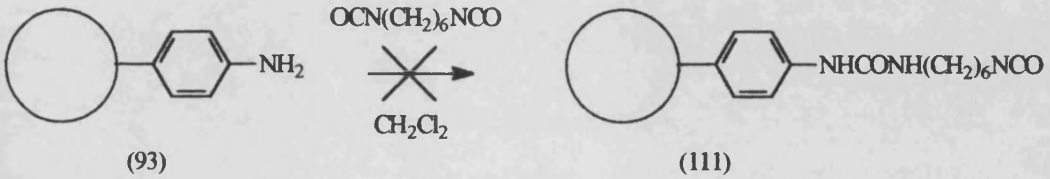



(106) (109) (110)

Scheme 5.16

A number of products were produced and the main product was isolated by column chromatography. NMR analysis of this compound showed the spacer to have cyclised to form the succinimide, rather than coupling to butylamine. This also makes this approach unsuitable for the coupling of porphyrin derivatives to peptide sequences.

Owing to the problems with cyclisation, longer spacers were investigated for which cyclisation onto the phenyl amino would be unfavourable. A joint coupling and activation procedure was first investigated. The aminoporphyrin was treated with an excess of 1,6-diisocyanatohexane. This was intended to provide a urea linkage onto the porphyrin and a highly reactive isocyanate for coupling to the peptide (Scheme 5.17).

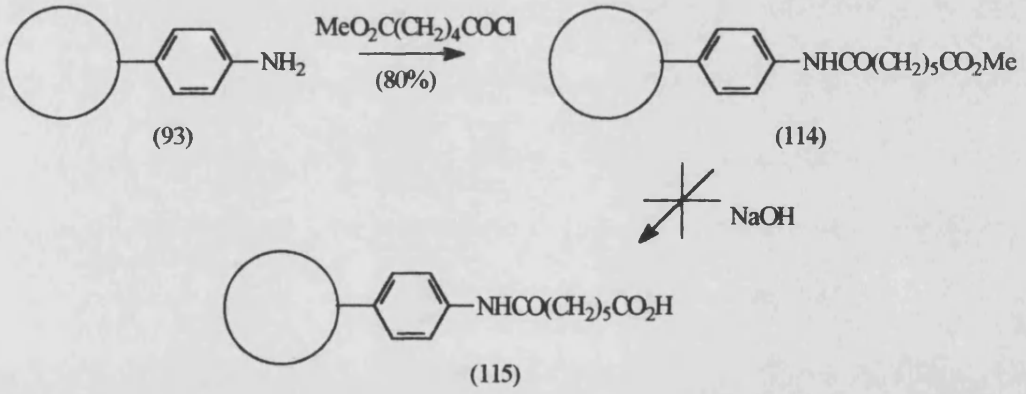



(93) (111)

Scheme 5.17

The coupling reaction proceeded very slowly, only after 5 days was complete conversion of the starting material observed. Distillation of the excess spacer revealed an intractable solid. This is presumably a bis-porphyrin formed by the reaction of both isocyanate groups with porphyrin amines.

An acid chloride approach was then utilised. Hexanedioic acid monomethyl ester in $CH_2Cl_2$ was treated with oxalyl chloride for 18 hours (Scheme 5.18). Formation of the acid chloride was confirmed by the IR spectrum (absence of an OH stretch). The crude material was then added to a solution of the aminoporphyrin in $CH_2Cl_2$. After 24 hours, complete conversion to a single product was observed. Following column chromatography, this product was confirmed, by NMR and MS, to be the desired methyl ester porphyrin derivative. In order to achieve coupling to a peptide the methyl ester must first be removed and then the revealed carboxylic acid activated. Base hydrolysis proceeded slowly due to the lack of solubility of the starting material. Following heating at reflux, the crude material was isolated by addition of acid. This product was sparingly soluble in most solvents. Mass Spectral analysis showed this material to contain both the desired carboxylic acid and the methyl ester.

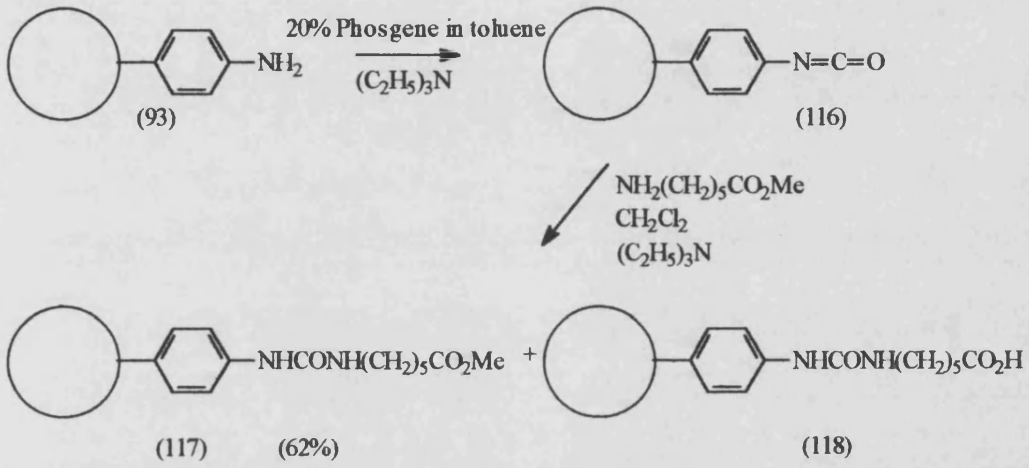



(93) (114) (115)

Scheme 5.18

In a further approach, 6-aminohexanoic acid was coupled directly to an activated porphyrin derivative (Scheme 5.19). The aminoporphyrin was converted to the isocyanate, on treatment with phosgene, and then allowed to react with methyl 6-aminohexanoate. After 18 hours, formation of two new products was observed, which were separated by column chromatography. NMR analysis showed the less polar

product to be the desired methyl ester and the more polar spot to be the carboxylic acid analogue. The presence of the carboxylic acid compound was probably due to an impurity in the starting ester, rather than loss of the ester during coupling. However, both of these products were sparingly soluble suggesting that hydrolysis, and subsequent activation would be difficult to achieve. Hence this approach seemed unsuitable for the coupling of porphyrin to peptide sequences.




Scheme 5.19

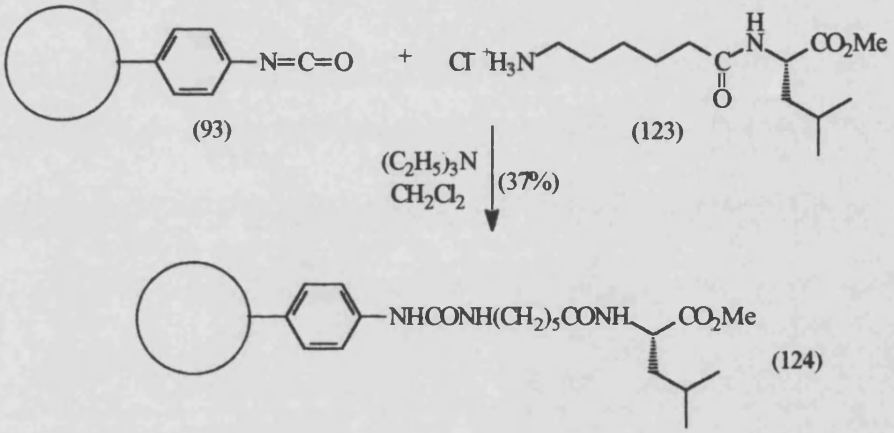
## 5.6 Non-spacer derivatives

A new synthetic approach was developed in which the peptide lysine or glutamic acid residues were extended prior to coupling with a non-extended porphyrin. Extension of both residues with 6-aminohexanoic acid derivatives would result in peptide derivatives containing both amino and carboxylic acid groups. Hence, coupling procedures for the porphyrin to both types of functionalisation are required.

### 5.6.1 Coupling to amines

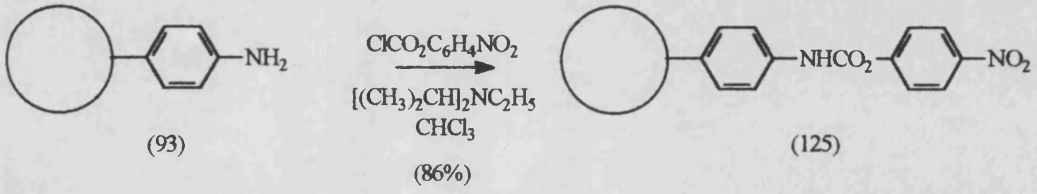
In order to develop a method of coupling to the amino peptide derivative, a model compound, N-(6-aminohexanoyl)leucine methyl ester hydrochloride, was prepared. Activation of the porphyrin as the isocyanate had proved largely successful in the

coupling with methyl 6-aminohexanoate. Thus, the isocyanate derivative was prepared and allowed to react with the new model compound (Scheme 5.20). After 24 hours, column chromatography gave compound **(124)** in 37 % yield. However, in an attempt to scale up this reaction, it proved difficult to isolate the desired compound by both column and preparative thin layer chromatography. In both these cases, a second product is formed during the activation procedure. This corresponded to N,N'-bis(tetraphenylporphyrinyl)urea, in which the porphyrin amine reacts with porphyrin isocyanate in preference to phosgene. Thus, as no purification of the activated porphyrin is undertaken, the crude material is of unknown purity.




Scheme 5.20

Therefore, it was proposed to use an isocyanate synthon, the corresponding 4-nitrophenyl carbamate, in the coupling reaction. This is relatively stable and can be purified prior to addition to the peptide, enabling a more controlled coupling reaction. Treatment of aminoporphyrin with bis-(4-nitrophenyl)carbonate proved unsuccessful, only slow conversion was observed over many days. Alternatively, treatment with the more reactive 4-nitrophenol chloroformate gave the porphyrin carbamate in 18 hours (Scheme 5.21).




Scheme 5.21

Column chromatography allowed the isolation of the pure product in 86% yield. This compound was then allowed to react with numerous peptide sequences as described in chapter 7.

5.6.2 Coupling to carboxylic acid groups

With glutamic acid peptides, incorporation of the spacer leads to formation of a γ-acylazide. This activation procedure will be described in chapter 7. Attempts to couple this to aminoporphyrin, a reaction which can be considered straightforward, resulted in unacceptably low yields. These results, coupled with the difficulties encountered with addition of spacers to aminoporphyrin, suggested that the amine is of low nucleophilicity. This has also been suggested by other workers. In the preparation of porphyrin dimers, Lindsey *et al* [252] found that the reaction of aminoporphyrin with a pentafluorophenyl porphyrin derivative gave very low yields. However, by using the more reactive aminomethylporphyrin, 81% yield of dimers was achieved. This was also the case with proline derivatives of aminoporphyrin, where the aromatic amine is altered to a rigid aliphatic type amine .

In order to increase the reactivity of the aminoporphyrin, glycine was coupled to the porphyrin. Gribkova *et al* [256] used both a mixed anhydride and direct DCC coupling approaches to attach both tyrosine and tryptophan to aminoporphyrin. Yields of 75-80% were achieved using the DCC coupling technique; however, the dicyclohexylurea was difficult to remove from the products [255].

Thus, glycine was coupled to the aminoporphyrin by an active ester reaction. N-Bocglycine pentafluorophenyl ester was prepared from the protected amino acid and pentafluorophenol by a DCC method. Initially, coupling of the amino acid to the porphyrin was attempted at room temperature. The reaction proceeded slowly and required further addition of the active ester to reach completion. Column chromatography proved unsuccessful in the separation of the desired porphyrin derivative from excess amino acid ester. However, heating a two fold molar excess of

# CHAPTER SIX

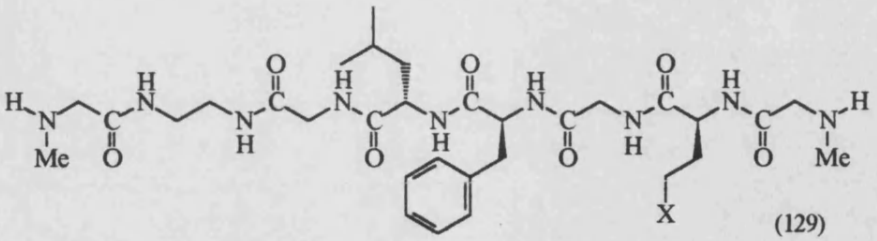
## PEPTIDE SYNTHESIS

### 6.1 Introduction

In order to prepare a linear degradable polymer based on PEG, a degradable peptide sequence is required, which can be incorporated into the polymer. The potential of the peptide sequence GlyPheLeuGly to be used as a lysosomally degradable sequence has been demonstrated in both pendant and cross-link situations. However, it has not been used to provide degradability of a linear polymer chain. Ulbrich [78] has prepared a PEG co-polymer where an amino acid, Phe, is incorporated in chain and imparts degradability. In this case, however, there is no functionalisation of the polymer to allow for attachment of drugs or imaging agents. Thus, in this project, a number of peptide monomers have been prepared based on the sequence GlyPheLeuGly but also incorporating at least one amino acid which can be used to couple a drug molecule to the polymer, for example glutamic acid and lysine.

The polymerisation reaction was designed to be between PEG activated as α,ω-bis-epoxide and a α,ω-bis-amine derivative of the peptide sequence. To afford an easy polymerisation, the same degree of reactivity is required at each terminus of the peptide monomer. Furthermore, to avoid cross-linking of the polymer, a secondary amine, which can only react once with the activated PEG, is required. The non-proteiogenic amino acid sarcosine (N-methylglycine) can be used to provide the secondary amine. This can be easily attached at the N-terminus of a peptide sequence by simple extension of the chain. To incorporate this amino acid at the C-terminus, however, requires the inclusion of a retro-inverso unit to change both the sense of the peptide chain and also the functionality. Of the many α,ω-bis-amines which are suitable for use as a retro-inverso unit, the small ethane-1,2-diamine unit was investigated.

Using this strategy a bis amine potentially degradable monomer suitable for inclusion in a polymer, can be prepared (Figure 6.1).



(129)

X = $CO_2H$ (Glutamic acid)
$(CH_2)_2NH_2$ (Lysine)

Figure 6.1

## 6.2 Peptide synthesis

In order to prepare a single dipeptide from two different amino acids, one must have a protected amino group and one, on the whole, should be activated at the carboxylic position. If no protection is in place, the single amino acid can couple to itself to give an unwanted dipeptide or polymers can be formed containing one or both amino acids.

### 6.2.1 Amino Protection

To protect the amine of an amino acid either its nucleophilicity should be suppressed by the use of an electron withdrawing substituent, or it should be sterically protected by the attachment of bulky substituents [264].

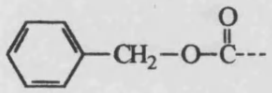
In the early days of solution phase peptide synthesis, simple N-acetyl and N-benzoyl protection were investigated. Although these afforded efficient protection for peptide synthesis, harsh conditions are required for the removal of these groups. The strong acids and bases used also lead to the destruction of peptide bonds and the collapse of the peptide [265].

Alkyloxycarbonyl derivatives, known as urethanes, were then investigated. These provide effective protection without racemisation and can be removed in conditions that do not affect the peptide bonds. In this situation, acyl-oxygen fission, to yield the free amine of the peptide, is very difficult to achieve. However, alkyl oxygen fission can occur easily in urethanes through a variety of mechanisms. This results in the formation of carbamic acids. These are highly unstable and undergo decarboxylation to yield the unprotected peptide [264,265].

Numerous urethanes have been developed, each with specific deprotection properties to enable the preparation of peptide sequences. In order to prepare a sequence, it is often essential to protect not only the $N^{\alpha}$ position of the amino acid but also reactive groups in the side chains. The main side chain functional groups requiring protection are the $N^{\varepsilon}$ of lysine, the $CO_2H^{\gamma}$ of glutamic acid, the OH of tyrosine and the SH of cysteine. Selective protection of these groups is often required in order to allow deprotection at one position in the peptide whilst retaining protection at other positions. Therefore, in designing a strategy for the preparation of a peptide sequence there is often a need for orthogonal protecting groups. There are two types of orthogonal protecting groups. Firstly, with truly orthogonal protecting groups, both groups can be removed without removing the other. Alternatively, partially orthogonal groups can be used in some cases. In this case the first group can be removed whilst the second is retained, whereas removal of the second group also results in the removal of the first [264,265].

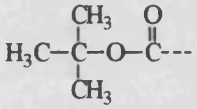
The main urethane protecting groups are truly orthogonal. The original benzyloxycarbonyl (Z) group, reported by Bergman and Zervas in 1932, is stable to mild acid and base but can be removed on treatment with hydrogen bromide in glacial acetic acid (HBr / Acetic acid) or by catalytic hydogenolysis. Boc, t-butoxy carbonyl, in contrast, is unaffected by catalytic treatment with hydrogen but is easily removed by acids such as trifluoroacetic acid (TFA) which leaves the Z group unharmed. The properties of these two protecting groups have led to their widespread use in peptide synthesis. More recently, a series of urethanes has been prepared which can be removed by treatment with base, through a proton extraction mechanism [264,265]. The

most commonly used example is Fmoc, 9-fluorenylmethyloxycarbonyl. This is stable in both weak and strong acids and therefore can be retained during acidic deprotections of Z and Boc groups. It is, however, susceptible to hydogenolysis so is not truly orthogonal to the Z group. Fmoc, itself, can be removed by treatment with secondary amines such as piperidine, which leave both Z and Boc unharmed. The introduction of this protecting group has lead to greater versatility in peptide synthesis strategies especially using solid phase methods [264,265].



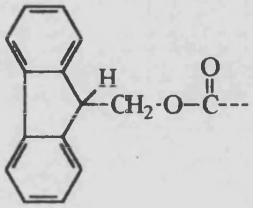
Benzyloxycarbonyl
(Z)

(130)



*t* Butoxycarbonyl
(Boc)

(131)



9-Fluorenylmethoxycarbonyl
(Fmoc)

(132)

Figure 6.2

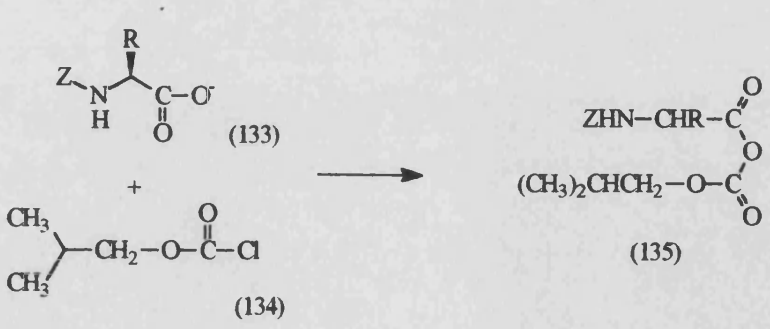
Protection of side-chain functional groups, apart from the amino groups of lysine and ornithine, is residue-specific. Methods are available for the selective protection of all amino acids. These are unimportant, however, within the context of this work.

### 6.2.2 Carboxyl Activation and Coupling

To achieve coupling of amino acids to peptides or other amino acids, it is usually necessary to activate the carboxylic acid moiety of the protected amino acid. Fischer [264] used acid chlorides to achieve amino acid coupling. This is an attractive technique as the reactions proceed very rapidly. However, because the conditions required to form the acid chlorides are harsh and acid chlorides are prone to racemization, this method is now only rarely used. There has been an increase in interest in this method more recently, since it was discovered that the acid chlorides of Fmoc protected amino acids are both easily prepared and relatively stable [265].

Curtius presented a coupling method based on acid azides. This is an attractive method as it appears to take place without racemization. Furthermore, esters of amino acids can be converted to hydrazides and hence azides without formal ester hydrolysis to the carboxylic acid. The major difficulty with this approach is the tendency of the azide to undergo intramolecular rearrangement to the isocyanate. This rearrangement can lead to peptide derivatives where the amino acid residues are linked *via* urea rather than peptide bonds. These peptide-like compounds are difficult to separate from the desired peptide. This azide coupling method will be described in more detail in Chapter 7 [264,265].

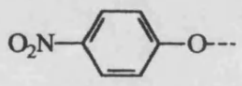
More recently anhydrides of amino acids have been prepared and used in peptide synthesis. Two types of anhydrides can be prepared. Firstly unsymmetrical or mixed anhydrides can be used. In this case the anhydride is formed between the amino acid and an organic acid. Therefore treatment of a peptide with a mixed anhydride can result in acylation of the peptide by both the amino acid and organic acid. This is particularly the case where the organic acid is benzoic acid as both the electrophilic sites have the same reactivity. This can be overcome by using half esters of carbonic acids to form the anhydride. The inclusion of an extra oxygen atom in this component reduces its reactivity and thus the major reaction is the acylation of the peptide by the amino acid. Mixed anhydrides, formed on treatment of the N-protected amino acid by ethyl chloroformate or isobutyl chloroformate, have been widely used in both peptide synthesis and in the attachment of drug molecule to peptides [264,265].



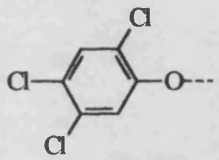

Scheme 6.1 : Mixed Anhydride

Symmetrical anhydrides are formed from two molecules of the amino acid. Thus, each component has the same reactivity and only one product can be formed. However, this approach is inherently wasteful, as one molecule of the protected amino acid is lost on each coupling [264,265].

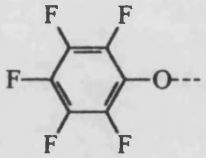
An alternative activation procedure is the formation of active esters. The majority of these active esters are phenyl esters. The effectiveness of these esters is dependent on the electron-withdrawing properties of the ring *i.e.* on the ring substituents. Initially, nitro substituted phenyl esters were investigated; this led to the development and extensive use of *p*-nitrophenyl esters of amino acids in peptide synthesis. Kupryszewski investigated pentachlorophenyl esters and found them to be highly reactive [265]. However, because the chloro groups are bulky, steric hindrance can lead to decreased reactivity in peptide couplings where there is steric interference. Pentafluorophenyl esters, however, are as active as the chloro derivatives but do not have the deleterious steric properties. 2,4,5-Trichlorophenyl esters are also highly effective, as the steric effects are reduced in comparison to the penta derivative [264,265].



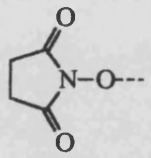
*p*-Nitrophenyl
(ONP)
(136)



2,4,5-Trichlorophenyl
(OTCP)
(137)



Pentafluorophenyl
(OPFP)
(138)

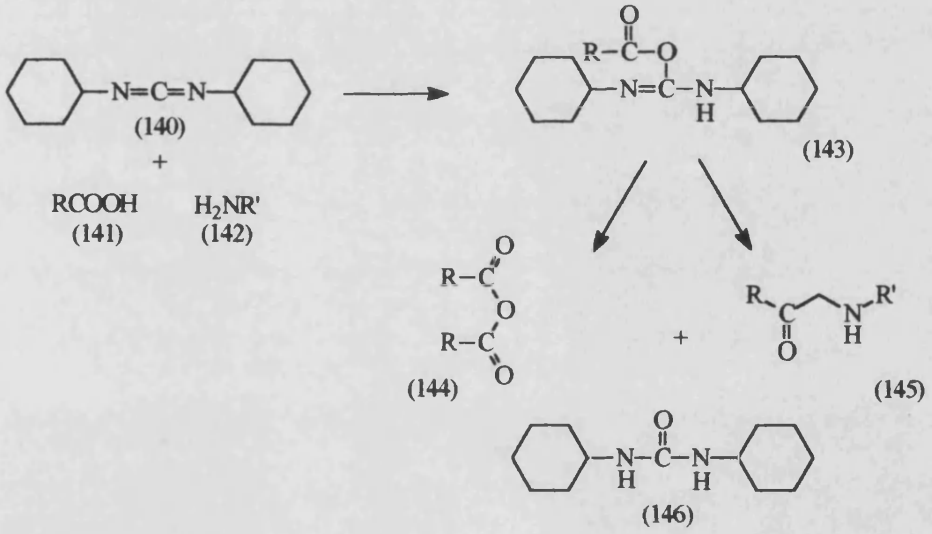


N-Hydroxysuccinimido
(ONSu)
(139)

Figure 6.3 : Active Esters

In a slightly different approach, active esters based on hydroxamic acids have been developed. In particular, N-hydroxysuccinimide has been widely used. It is attractive as, as well as having high leaving group reactivity, it confers enhanced water solubility on the amino acid. The activity of all these esters can be enhanced by addition of auxiliary nucleophiles to the coupling reaction. These compounds, such as 1-hydroxybenzotriazole (HOBT) and 4-dimethylaminopiperidine (DMAP), catalyse the aminolysis of the esters and can form more reactive electrophiles *in situ*.

The final approach to coupling does not rely on isolatable active forms of the amino acid. Direct condensation, leading to formation of the peptide bond can be achieved using condensing agents. The most widely used agent is dicyclohexylcarbodiimide (DCC), which was developed in 1955 by Sheenan and Hass. In this method, a conjugate of the amino acid to be coupled and DCC is formed which can then be broken either by coupling of the carbonyl to the amine, or by formation of a symmetrical anhydride (Scheme 6.2). Regardless of mechanism, the coupling procedure is highly effective.




Scheme 6.2

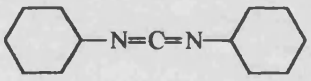
Two difficulties have been recognised in the use of this approach. Firstly, once coupling has taken place the condensing agent is transformed to dicyclohexylurea (DCU). This is almost insoluble in most organic solvents, so can be filtered from the

reaction mixture. However, a small portion can remain in solution and is thus difficult to remove. Secondly, intramolecular rearrangements can occur with the O-acyl conjugate. A N-acyl urea is formed which cannot react further to allow coupling and is also difficult to remove from the peptide as it is freely soluble (Scheme 6.3).

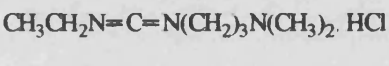
(143) (147)

Scheme 6.3

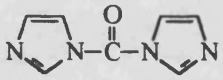
Other condensing agents have also been developed including water soluble carbodiimide and carbonyldiimidazole (CDI). Apart from acting as a condensing agent in coupling reactions, DCC has been used for the formation of active ester and symmetrical anhydride derivatives of amino acids.



Dicyclohexylcarbodiimide
DCC
(140)



'Water soluble carbodiimide'
WSCI
(148)



Carbonyldiimidazole
CDI
(149)

Figure 6.4 : Coupling Reagents

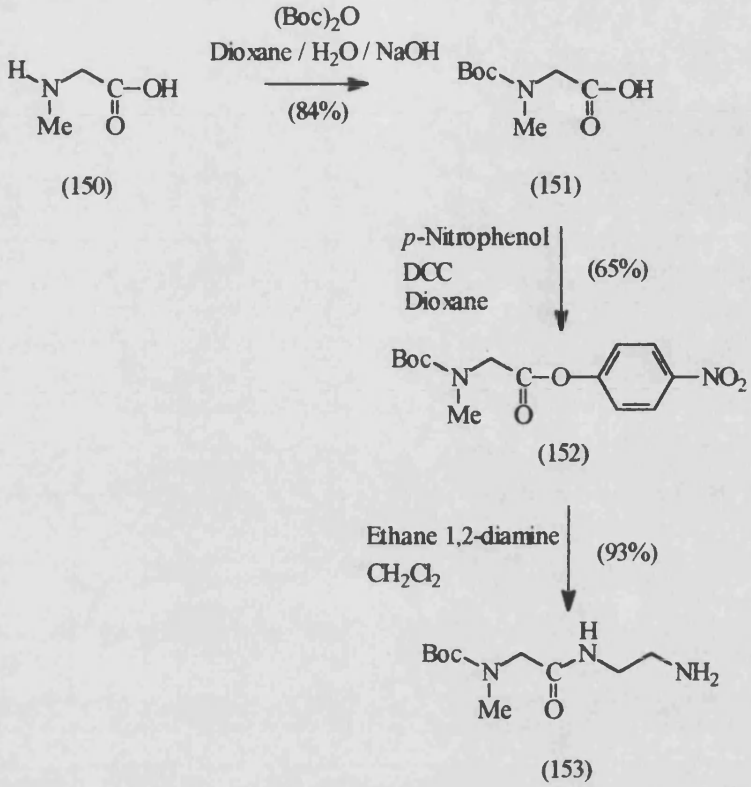
## 6.3 Non-degradable monomers

### 6.3.1 Boc and Fmoc protecting group strategy

In the first place, small peptides were prepared to test the suitability of the strategy and to develop protection tactics for preparation of the degradable monomers. These are built up of only a functionalised amino acid, glutamic acid or lysine, rather than the degradable sequence.

Two protecting groups are required in the synthesis of these monomers. One is necessary for the long-term protection of sarcosine and one for the temporary protection of the functionalised amino acid. Obviously the protecting group for the functionalised amino acid must be removed selectively in the presence of the sarcosine protecting group. Thus, the orthogonal protecting groups Fmoc and Boc were initially proposed. Fmoc was chosen as the temporary protecting group and Boc as the more permanent.

Boc-sarcosine was easily prepared using a modification of the method of Anderson and MacGregor [266] (Scheme 6.4). Acylation was achieved in a basic dioxane / water mixture by application of di-*t*-butyl dicarbonate $(Boc)_2O$. Some workers have suggested [267] that Boc protecting groups can be lost during work up procedures with strong inorganic acids. Thus they suggest that the weaker citric acid should be used. However, result in this laboratory [268] have demonstrated that the protection can be retained during brief treatments of the Boc-amino acids in immiscible organic solvent with cold 10% aqueous sulphuric acid.




Scheme 6.4

Throughout this work, peptide couplings are achieved using active ester procedures. Active esters were prepared by treatment of the amino acid with the substituted phenol derivative and DCC in a suitable organic solvent. Thus BocSarONP was prepared by treatment of compound **(151)** with *p*-nitrophenol.

In order to prepare a mono sarcosyl derivative of ethane-1,2-diamine, the activated amino acid was added to a 20 fold excess of ethane-1,2-diamine in $CH_2Cl_2$. This approach enabled the isolation of the mono acyl compound **(153)** in 93% yield. No di acyl compound was isolated.

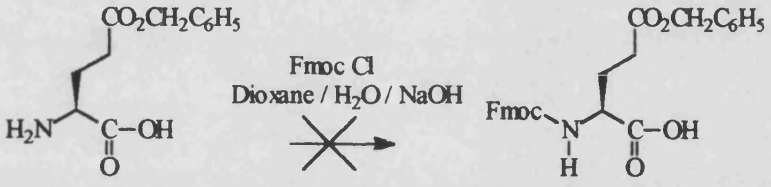
6.3.1.1 Protection of glutamic acid

Glutamic acid has two carboxylic acid functions and, thus, on activation, would provide a mixture of activated products which could take part in peptide couplings. To avoid this occurring, the γ-carboxylic acid group must be protected. This is most

easily achieved using esters and a number of γ-protected glutamic acid derivatives are available. Initially, the γ-benzyl ester was chosen as this protecting group has deprotection strategies which are orthogonal to both Fmoc and Boc.

Fmoc was an attractive candidate for the $N^{\alpha}$ amino protection as it is both fully orthogonal to Boc and can usually be easily deprotected. Brief treatment of the Fmoc-amino acid with piperidine results in the formation of both the desired deprotected compound and a derivative of Fmoc, dibenzofulvene, which can be removed by filtration [269] Alternatively, ammonia, diethylamine and ethylamine can be used. Although these deprotections proceed more slowly, these methods have the added advantage that the base can be removed by evaporation [270].

In a first approach, attempts were made to incorporate the Fmoc protecting group using the method of Carpino and Han [269] (Scheme 6.5). A solution of fluorenylmethyl chloroformate was added slowly to a solution of the γ-benzyl glutamic acid in base. Unfortunately, coupling was not achieved with this method. NMR analysis of the organic washes showed the presence of 9-fluorenylmethanol. This suggests that either the desired product is formed but the protecting group is immediately cleaved or the chloroformate has decomposed.

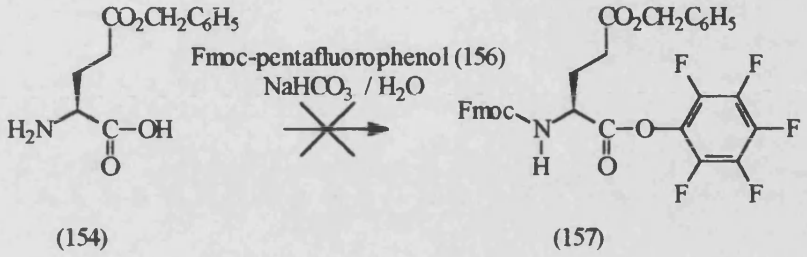



(154) (155)

Scheme 6.5

As the Fmoc-protected amino acid will be transformed to its active ester prior to coupling to the ethane-1,2-diamine derivative, protection and activation using the method of Schön and Kisfaludy [271] was investigated. This method enables formation of a protected, activated amino acid in a one-pot process. This approach has proved

successful with a large number of amino acids including the γ-tert butyl ester of glutamic acid [271]. However, this approach proved unsuccessful with the γ-benzyl derivative (Scheme 6.6). A similar approach using Fmoc-succinimide has also been used to prepare the protected derivative of γ-tert butyl ester of glutamic acid [272,273].




(154) (157)

Scheme 6.6

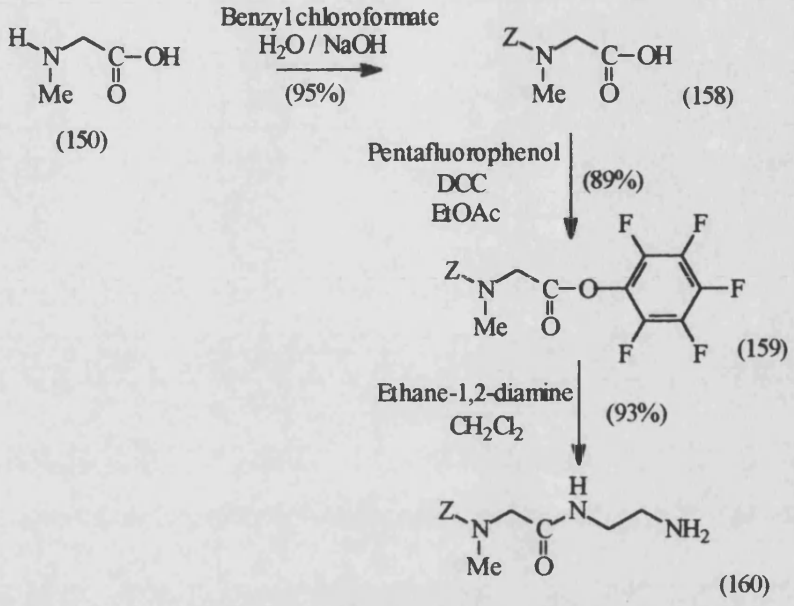
6.3.2 Boc and Z protecting group strategy

6.3.2.1 Glutamic acid monomers

As the Fmoc protection was difficult to achieve, a new synthetic strategy was developed. A new protecting group which is orthogonal to Boc was required. The obvious candidate is the Z group. This could be used to protect the glutamic acid, like Fmoc. However, the conditions required for removal of Z groups; HBr / Acetic acid or catalytic hydogenolysis make these reactions relatively difficult to perform. In comparison the removal of Boc is a fast and simple process. Thus, a new strategy was proposed in which Z was used as the protecting group for sarcosine and Boc for the incoming amino acid, and in the long term, for all amino acids in the GlyPheLeuGly sequence.

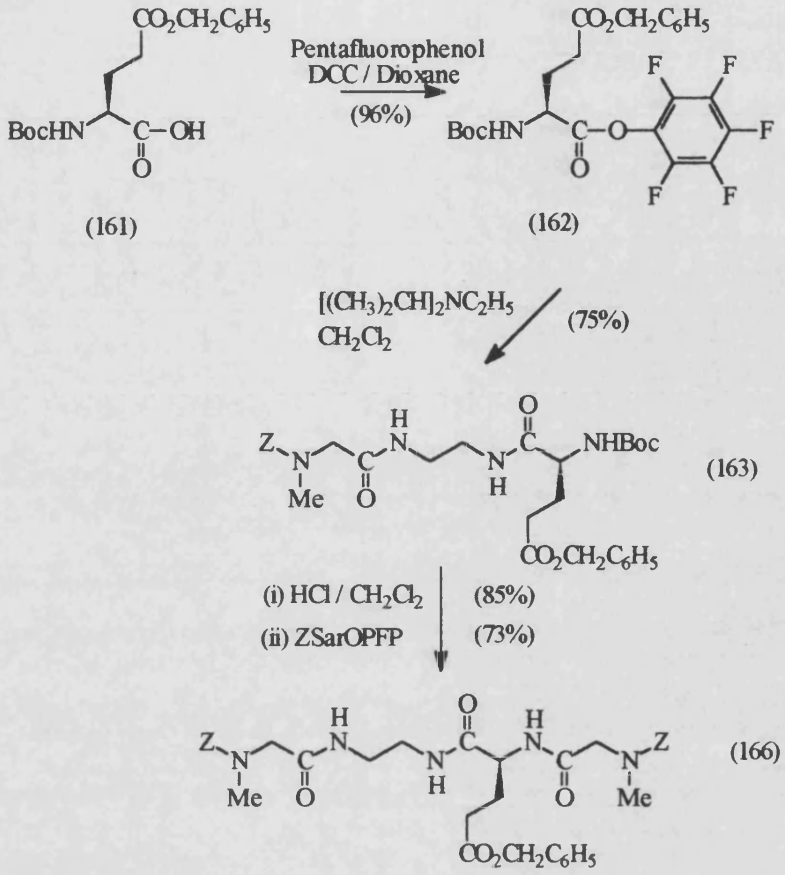
Z Sar **(158)** was easily prepared from sarcosine using benzyl chloroformate under Schotten Bauman conditions. This was then activated as the more reactive pentafluorophenyl ester and successfully coupled to ethane-1,2-diamine to afford ZSar-ethane-1,2-diamine unit **(160)** in 93% yield (Scheme 6.7). Boc-protected glutamic acid γ-benzyl ester is commercially available and was successfully activated

as the pentafluorophenyl ester and coupled to **(160)** yielding **(162)** (75%) (Scheme 6.8).




Scheme 6.7

Boc protecting groups are easily and rapidly removed on treatment with acids. The most common method involves the treatment of the peptide with TFA. This acts both as the solvent and reagent yielding the unprotected peptide as the trifluroacetic acid salt. However, TFA has been shown to cleave Z groups on prolonged exposure [265]. Previous experience in this laboratory has demonstrated that treatment of the peptide in an organic solvent with gaseous HCl yields the hydrochloride salt of the peptide. This is an attractive approach as the reaction is rapid and both the solvent and excess reagent can be easily removed by evaporation. Thus compound **(163)** in dioxane was treated with HCl to give the hydrochloride salt **(164)** in 85% yield.




Scheme 6.8

Coupling of the final amino acid, Z Sar, could prove difficult due to steric interference, especially with longer peptide sequences. Thus, an acid chloride coupling method was investigated for this step. The acid chloride was prepared by treatment of the protected amino acid with oxalyl chloride and DMF. On formation, the solvents and excess reagent were evaporated and the acid chloride added to an excess of a model amine, cyclopropylamine. Unfortunately, a number of products were formed in this coupling reaction. Thus, an active ester approach was utilised. ZSarOPFP coupled easily with compound **(164)** to give the bis-amine monomer **(166)** in 73% yield.

In the above situation, removal of the benzyl protecting group, to allow coupling of the porphyrin, or of the Z group, to allow polymerisation would result in the loss of

both protecting groups. Thus, three amines would be exposed which could result in branched polymers or the attachment of more than one porphyrin molecule. Although no differentiation is apparent using catalytic hydogenolysis deprotection, Ben Ishai [274] has demonstrated that, in some circumstances, the Z group can be preferentially removed on treatment with HBr / Acetic acid. Ideally a truly orthogonal protecting group would be preferable.

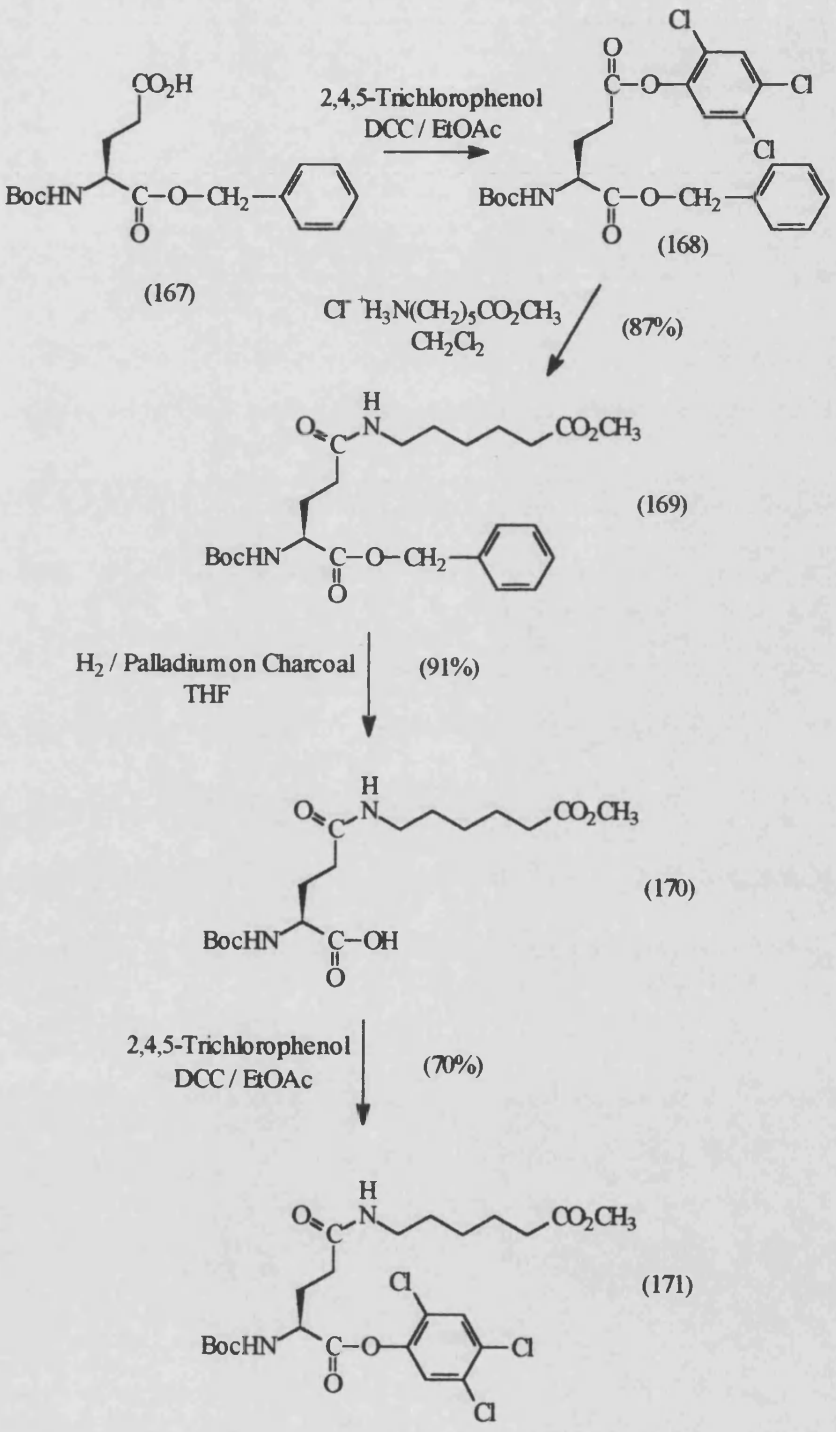
A commonly used ester protecting group is the tert-butyl group. However, this is not suitable as it is difficult to achieve selective removal of this group in the presence of a Boc group [265]. Interestingly, some workers have suggested that, with care, Boc can be removed in preference to the tert butyl ester by treatment with TFA in EtOAc.[275]

An attractive alternative is the methyl ester. This is unaffected by treatment with TFA, HCl, HBr / Acetic acid and catalytic hydrogenolysis and is therefore orthogonal to Z and Boc. However, if this amino acid derivative were to be incorporated into a peptide sequence, there would be a risk of pyroglutamate formation and subsequent loss of the methyl ester upon removal of the Boc protection [264,265]. As coupling of the porphyrin was found to be easier through an extended peptide derivative, an amide-linked spacer terminating in the Me ester was used to protect the γ-position. In this case pyroglutamate formation cannot occur.

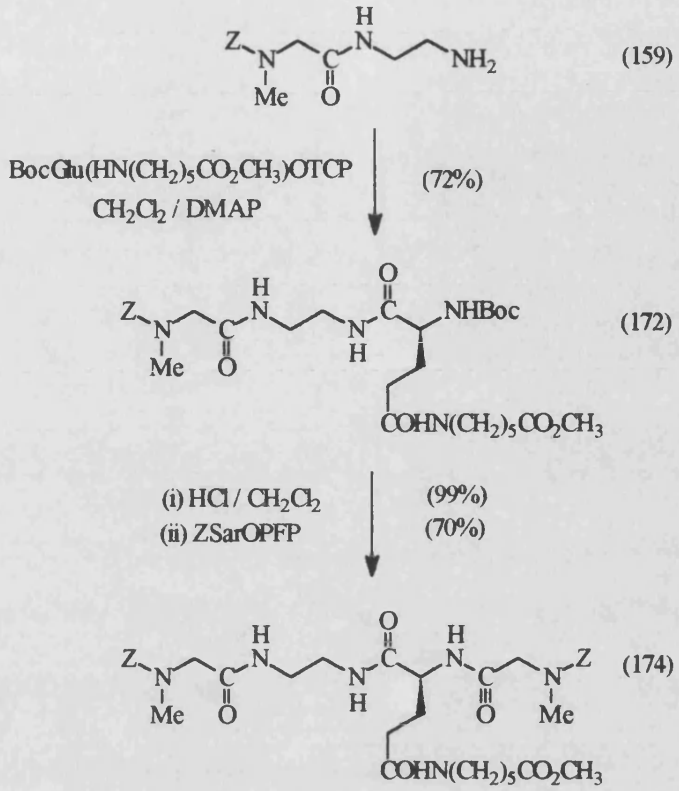
Boc-glutamic acid α benzyl ester was activated at the γ-position as the 2,4,5-trichlorophenyl ester (TCP ester). These esters are less reactive than pentafluorophenol esters; however, in situations where steric hindrance is minimal, satisfactory rates of coupling can be achieved. It also offers the more mundane advantage that it is less expensive, an important consideration in large scale synthesis. TCP esters can be prepared using the standard method, DCC in organic solvent. Alternatively an activated TCP derivative can be prepared which reacts directly with the amino acid [267].

This was successfully coupled with methyl 6-aminohexanoate to give the extended derivative, **(169)** in 87% yield. The α-benzyl ester was then removed by catalytic

hydrogenolysis in THF to give the free carboxylic acid. Treatment of this with TCPOH and DCC afforded the 2,4,5-trichlorophenyl ester **(171)**. This ester was coupled efficiently to the ethane-1,2-diamine and deprotected on treatment with HCl. ZsarOPFP was successfully coupled to give **(174)** in 70% yield.



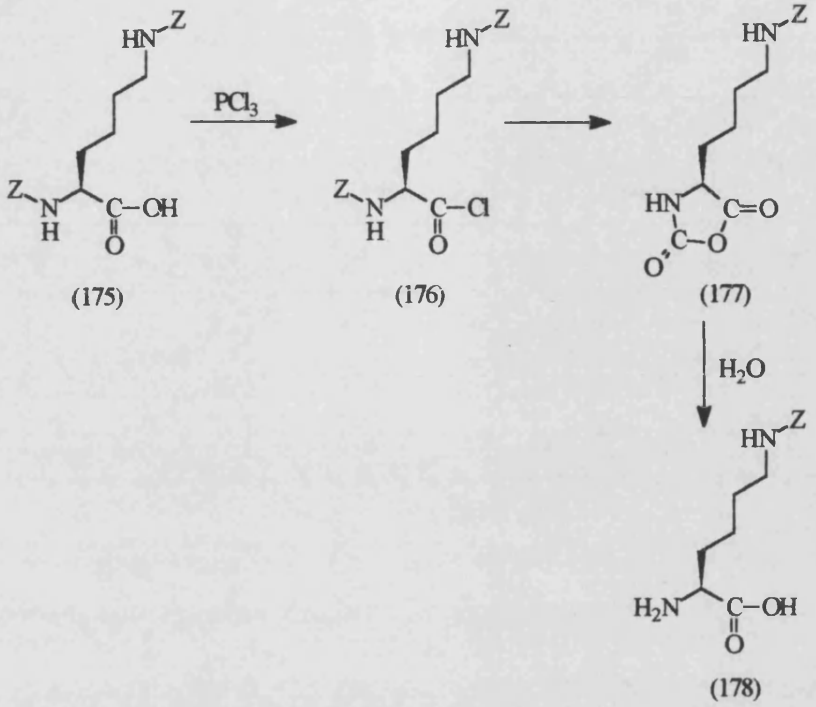

Scheme 6.9




Scheme 6.10

6.3.2.2 Lysine Monomer

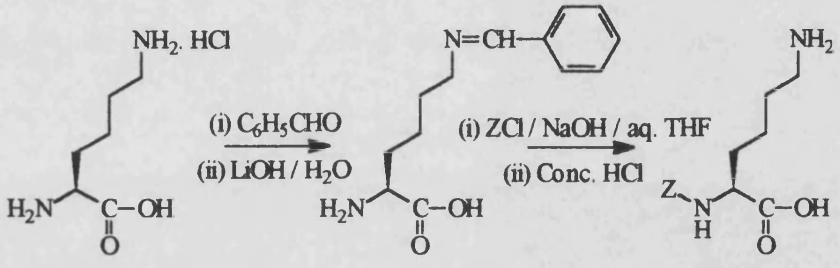
Lysine offers further opportunities in the design of an orthogonal protecting group strategy. The ε amino group has comparable reactivity to the α amino group so, if unprotected, could react with an activated amino acid to form a branched peptide [601]. There are four major approaches to differential protection of the amino groups of lysine. Bergman and Zervas proposed a method based on the selective deprotection of a di-Z-protected lysine using phosphorus trichloride and base [264]. (Scheme 6.11).




(175) (176) (177) (178)

Scheme 6.11

Alternatively the Z protecting group can be selectively introduced onto the α-position. Wünsh [265] developed a process in which the ε-amine is temporarily protected by treatment with benzaldehyde, allowing the Z group to be selectively introduced to the ε-amine. (Scheme 6.12)

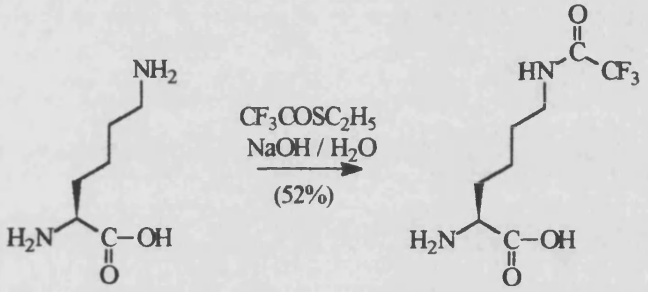



(175) (179) (180)

Scheme 6.12

A method, developed by Schallenberg and Calvin [276], relies on the different solubility characteristics of the α and ε amino protected compounds. Treatment of an amino

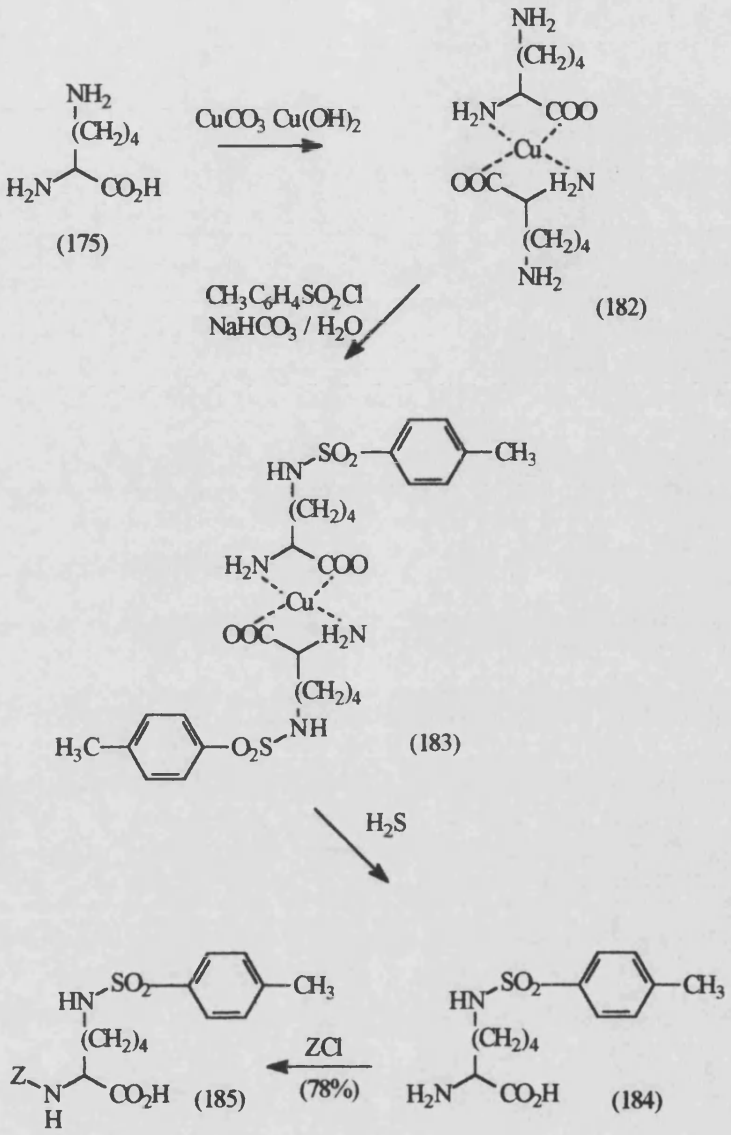
acid with ethylthioltrifluoroacetate in mildly basic conditions results in the formation of protected amino acid. This protecting group is stable in most conditions but can be cleaved by raising the pH to 10 either by treatment with aq. NaOH or conc. ammonia. However, for lysine, it is possible to isolate the ε-protected derivative alone in 52% yield. This is not thought to be due to a regio-specific mechanism as the α position of many amino acids can be selectively protected in this manner but is probably due to a lack of solubility of the ε-protected compound, allowing its isolation by crystallisation.(Scheme 6.13)




(175) (181)

Scheme 6.13

The most popular method of selective protection of lysine is based on the formation of copper (II) complexes. Addition of copper (II) carbonate to a solution of lysine results in a complex of two lysine residues and one $Cu^{2+}$ ion, in which the α-amine provides a ligand for the ion and is thus unavailable for reaction. The complex can then be treated with most protecting groups to afford the ε-amino protected compound. The complex can then be broken on treatment with $H_2S$ leaving a free α-amine which can be protected with an orthogonal protecting group if desired. One of the most common derivatives formed using this method is the α-Z-ε-tosyl lysine. (Scheme 6.14) [264].



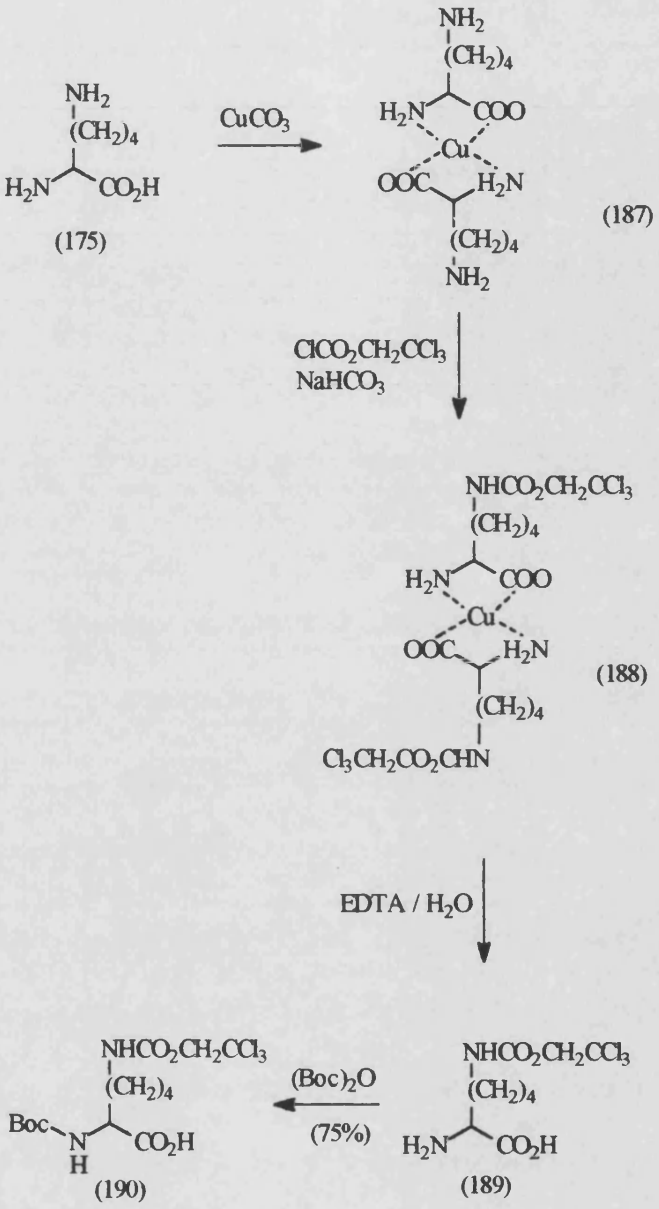

Scheme 6.14

Initially, attempts were made to prepare the ε-trifluoroacetyl derivative of lysine using the method of Schallenberg and Calvin [276]. This is an attractive protecting group as it is stable to treatment with HBr/Acetic acid and to conc. HCl. However it can be easily cleaved on treatment with inorganic base ($Na_2CO_3$, $K_2CO_3$) in methanol. The acetylating agent, ethylthioltrifluoroacetate, was easily prepared in 49% yield. Treatment of lysine with this reagent, following the method of Bodanszky [267], resulted in a low yield (7%) of a solid upon recrystallisation. This was not soluble in any of the

available NMR solvents and thus was not characterised. This low yield, lead to the development of a new approach.

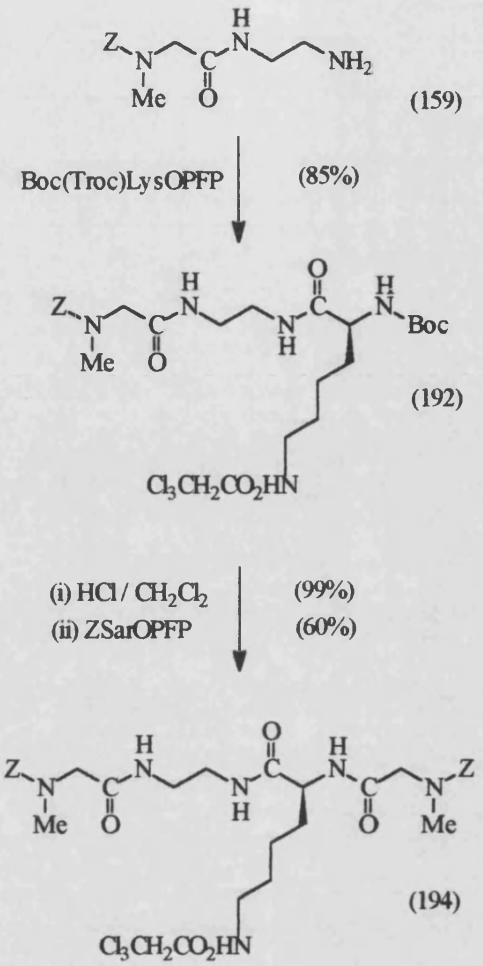
Copper complex methods were then investigated. The most widely used group, tosyl, is unsuitable for a Z / Boc protection strategy. Although it can be removed selectively by treatment with KOH in MeOH on heating, most other deprotection methods also result in the cleavage of Z or BOC for example, treatment with HF, HBr / Acetic acid or sodium in liquid ammonia [277]. After consideration, a less widely used protecting group was chosen for the protection of the ε-amine. 2,2,2-Trichloroethoxycarbonyl (Troc) protection can be easily introduced, via the chloroformate, to an amine. It is resistant to treatment with HCl and thus is orthogonal to Boc. Although it is resistant to treatment with HBr / Acetic acid, it is usually affected by catalytic hydrogenolysis. During hydrogenation, one or more chlorines can be replaced by hydrogen, thus rendering the protection permanent. It can be selectively cleaved by treatment with zinc or cadmium in a variety of solvents [264,265]. (Chapter 7).

The copper complex of lysine was formed easily following the method of Yajima *et al.* [278] The Troc group was successfully introduced using Schotten Baumann conditions. As discussed the complex can be easily broken with $H_2S$. However, as this gas is toxic, the same reaction can be achieved using thioacetamide in base. An alternative approach is to break the complex using the chelator EDTA. Using this latter approach the complex was easily broken. Treatment with $(Boc)_2O$ yielded **(190)** (75%) (Scheme 6.15). Unfortunately it was not possible to isolate the intermediate compounds.




Scheme 6.15

This diprotected amino acid was then successfully activated as the PFP ester. This was efficiently coupled to the ZSar-ethane-1,2-diamine unit yielding **(192)** (85%). The compound was then successfully deprotected, by treatment with HCl, without loss of the Troc protection. The final coupling of ZSarOPFP gave compound **(194)** in 60% yield.




Scheme 6.16

Thus simple monomers containing glutamic acid and lysine were successfully prepared. Thus proving both the suitability of the monomer preparation strategy and the potential for inclusion of a functionalised amino acid, with or without extension of the side chain. From this base, peptide sequences were prepared in which both a functionalised amino acid and the degradable sequence were present.

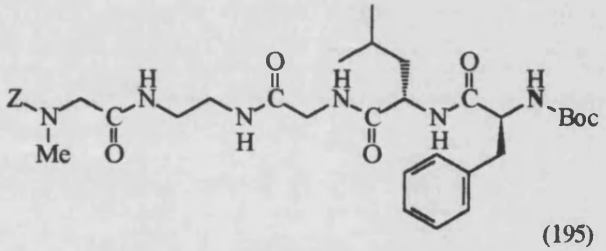
## 6.4 Degradable Monomers

### 6.4.1 Monomers with one functionalised amino acid

In the preparation of larger peptide sequences, two synthetic approaches can be used. A convergent synthesis where units of a few amino acids are condensed in a final step

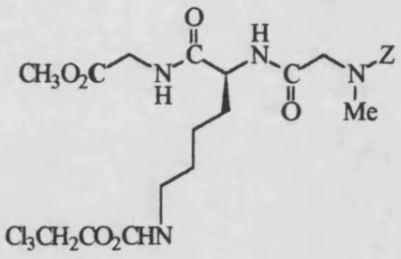
is particularly attractive. In this situation, the yield of the desired peptide is higher than if each amino acid is added to the growing peptide in a stepwise manner. It is also easier to isolate a final product formed using a convergent method as it is significantly different to the starting components. With a stepwise synthesis, it can be difficult to separate the desired peptide from peptidyl impurities which have a missing amino acid at some point in the sequence. However, it is essential when developing a convergent approach to choose the position of final condensation carefully so as to minimise racemisation and enable straightforward synthesis [265].

Initially, the peptide monomer incorporating lysine and the biodegradable unit GlyPheLeuGly was synthesised. In this synthesis, a convergent approach was first investigated. Two target peptides were designed, one being the tripeptide SarLysGly **(196)**, the other being PheLeuGly attached to the ZSar-ethane-1,2-diamine unit **(195)**. Therefore, the final condensation between an activated Gly derivative and Phe should be relatively simple.



(195)

N-Portion



(196)

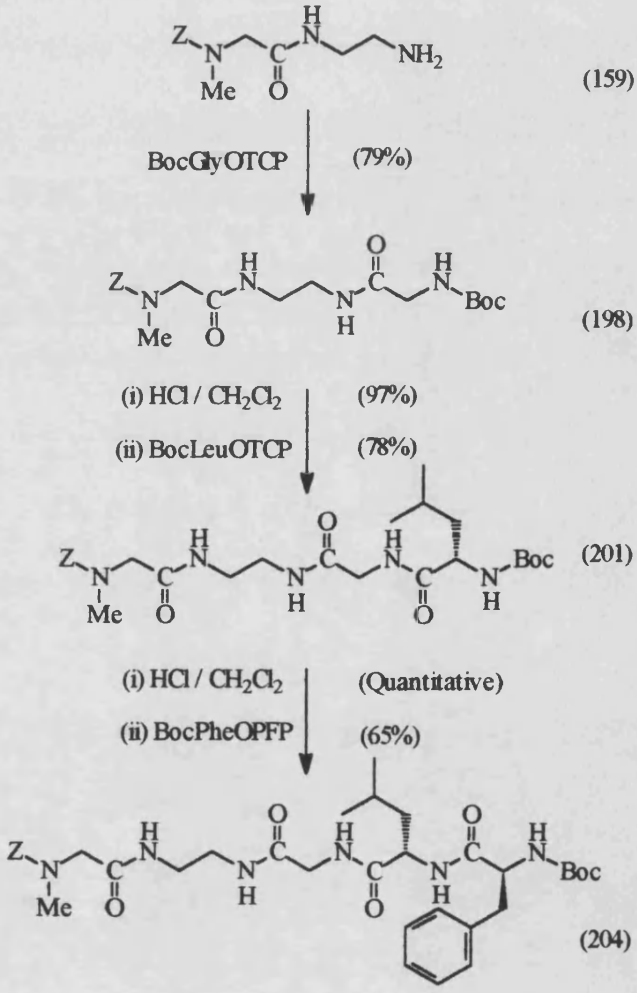
C-Portion

Figure 6.5

For this synthesis, the active esters of all the commercial Boc protected amino acids were prepared using the standard DCC coupling approach. For all amino acids, except

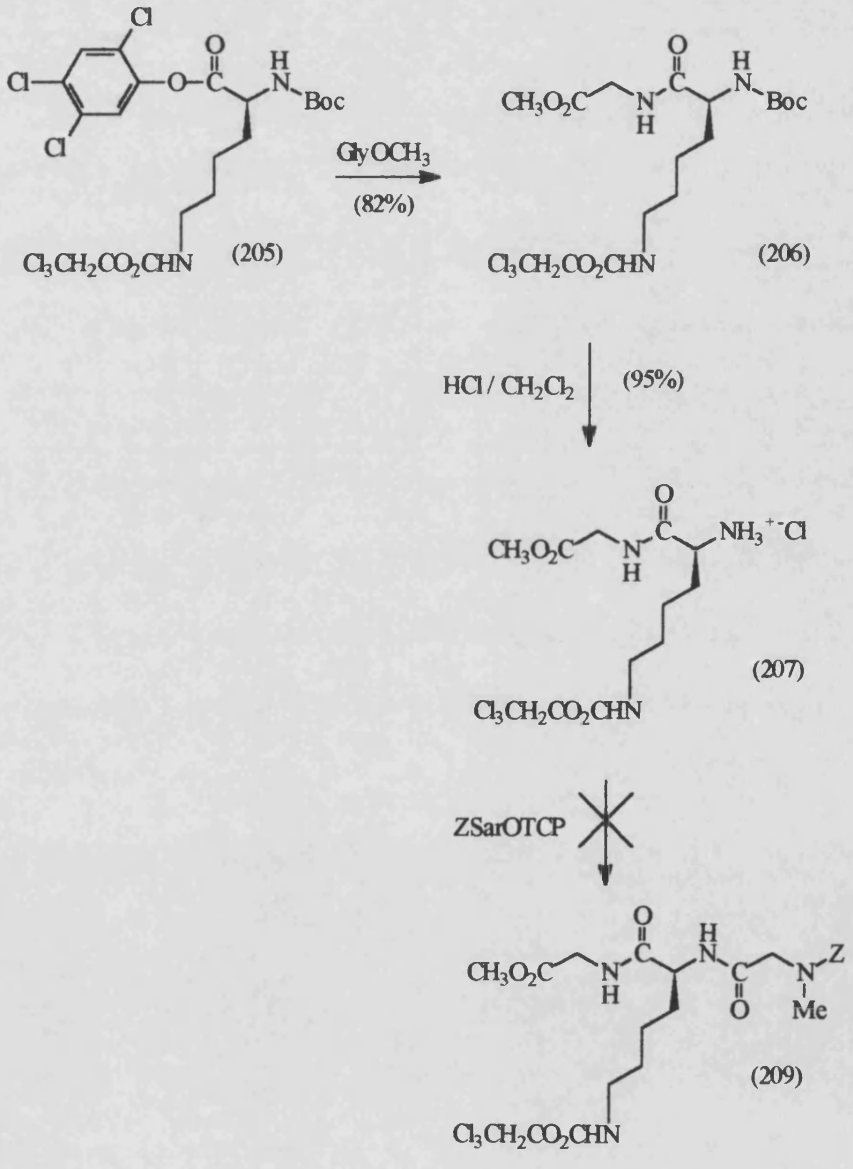
Phe, active esters of TCP were used. For Phe, it has been previously found that couplings proceeded slowly, possibly due to steric effects [268]. As this would be compounded in this situation where a coupling occurs between Phe and Leu, Phe was activated as the more reactive PFP ester.

Synthesis of the C terminus portion proceeded swiftly. Coupling of glycine to the ZSar-ethane-1,2-diamine unit was rapid, resulting in the formation of **(198)** in 79% yield. This was easily deprotected, by treatment with HCl, and coupled to BocLeuOTCP. Subsequently, this peptide was deprotected and coupled to the activated Phe, yielding **(204)** (65%).



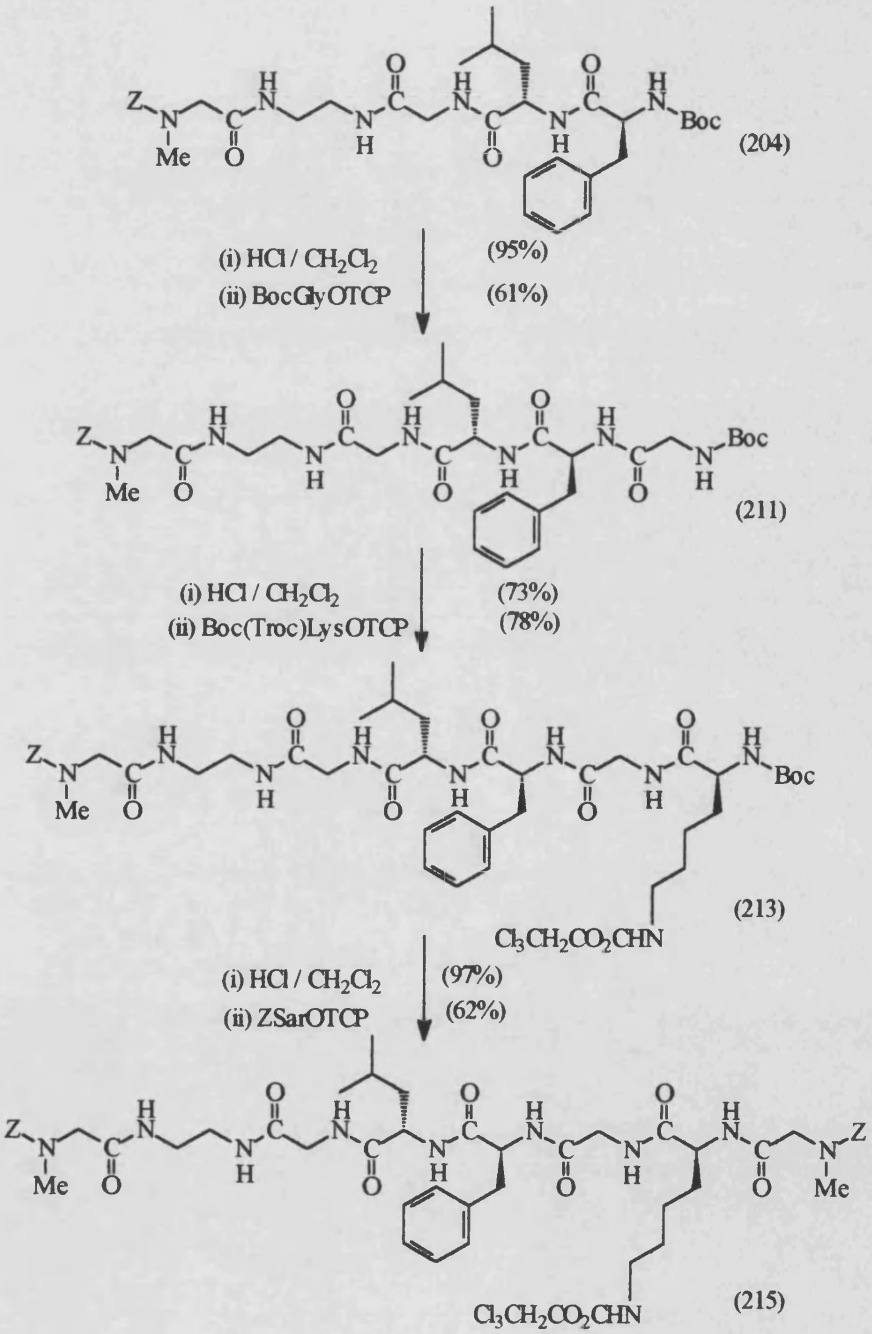

Scheme 6.17

Preparation of the N-terminal portion was more complex. Firstly a carboxyl-protected derivative of glycine was required. The simple methyl ester was chosen as it is commercially available. This reacted successfully with the TCP ester of $N^{\alpha}$-Boc-$N^{\varepsilon}$-TrocLysine to give the dipeptide in 82% yield (Scheme 6.18). The Boc protection was removed. Coupling of this compound with ZSarOPFP, however, proved problematic. Numerous products were formed and isolated by column chromatography. They were however unidentifiable. This is probably due to a loss of one or more of the protecting groups (Me ester or Troc) during the coupling reaction.



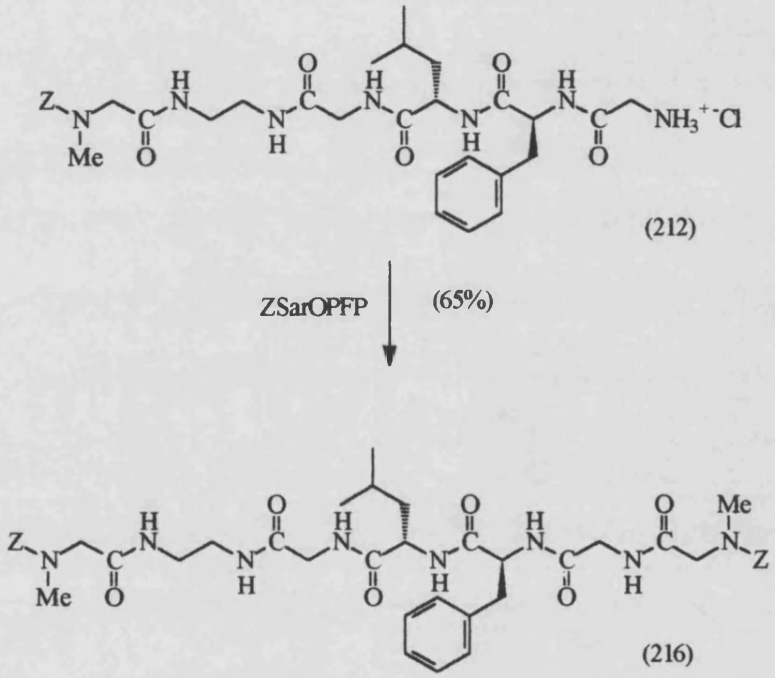

Scheme 6.18

From this, it was decided to abandon the convergent approach in favour of a stepwise approach. As a tripeptide C-terminal portion had already been prepared, this was deprotected and allowed to couple sequentially with the Boc-protected active esters of Gly and $N^{\varepsilon}$-protected-Lys. Final deprotection, followed by coupling of ZSarOPFP, gave the desired peptide monomer, **(215)** in 62% yield (Scheme 6.19).



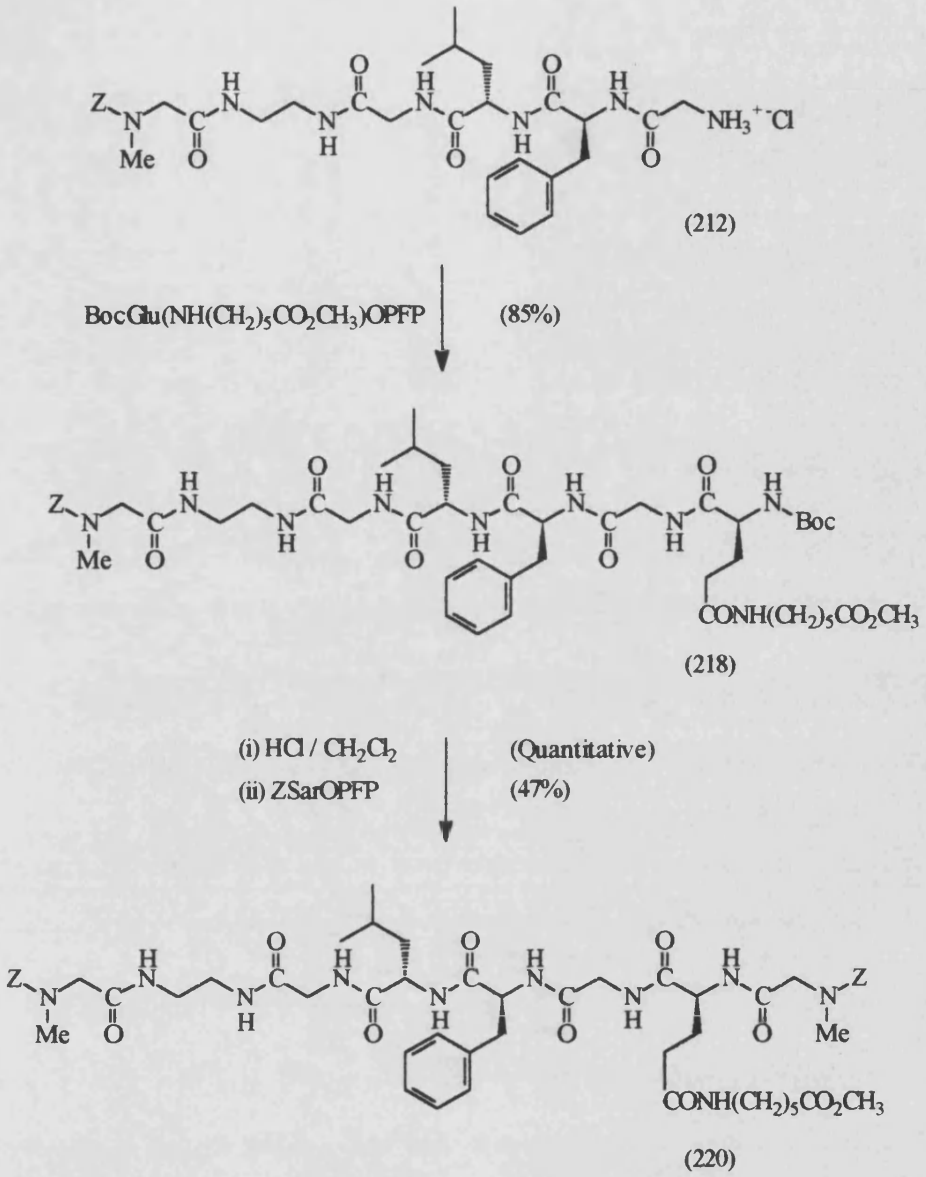

Scheme 6.19

From this basis, two further peptide monomers could be prepared. Firstly a monomer consisting of the degradable sequence GlyPheLeuGly and no functionalised amino acid and secondly, one containing the degradable sequence and the extended glutamic acid. Thus, it is apparent that a stepwise synthesis is advantageous for the synthesis of monomers, as it is possible to prepare three separate monomers in at the most four simple steps from one precursor. The GlyPheLeuGly monomer **(216)** was easily prepared in 65% yield by coupling of ZSarOPFP to **(212)**.




Scheme 6.20

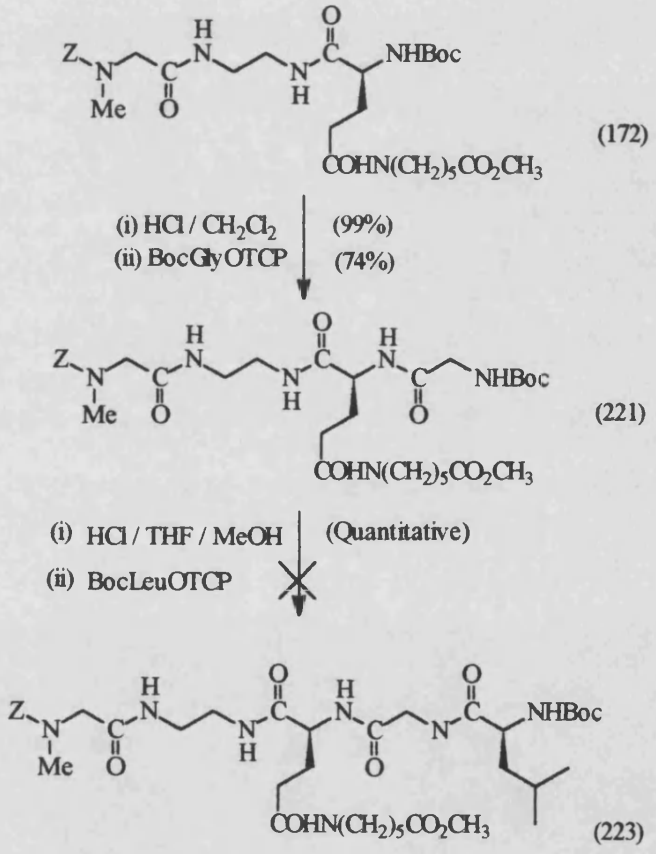
With the glutamic acid derivative, coupling of the derivatised amino acid enabled isolation of the peptides as solids, on trituration with ether. However, higher yields of the peptides were obtained using column chromatography. Thus, the desired monomer, **(220)** was obtained in 47% yield (Scheme 6.21).




Scheme 6.21

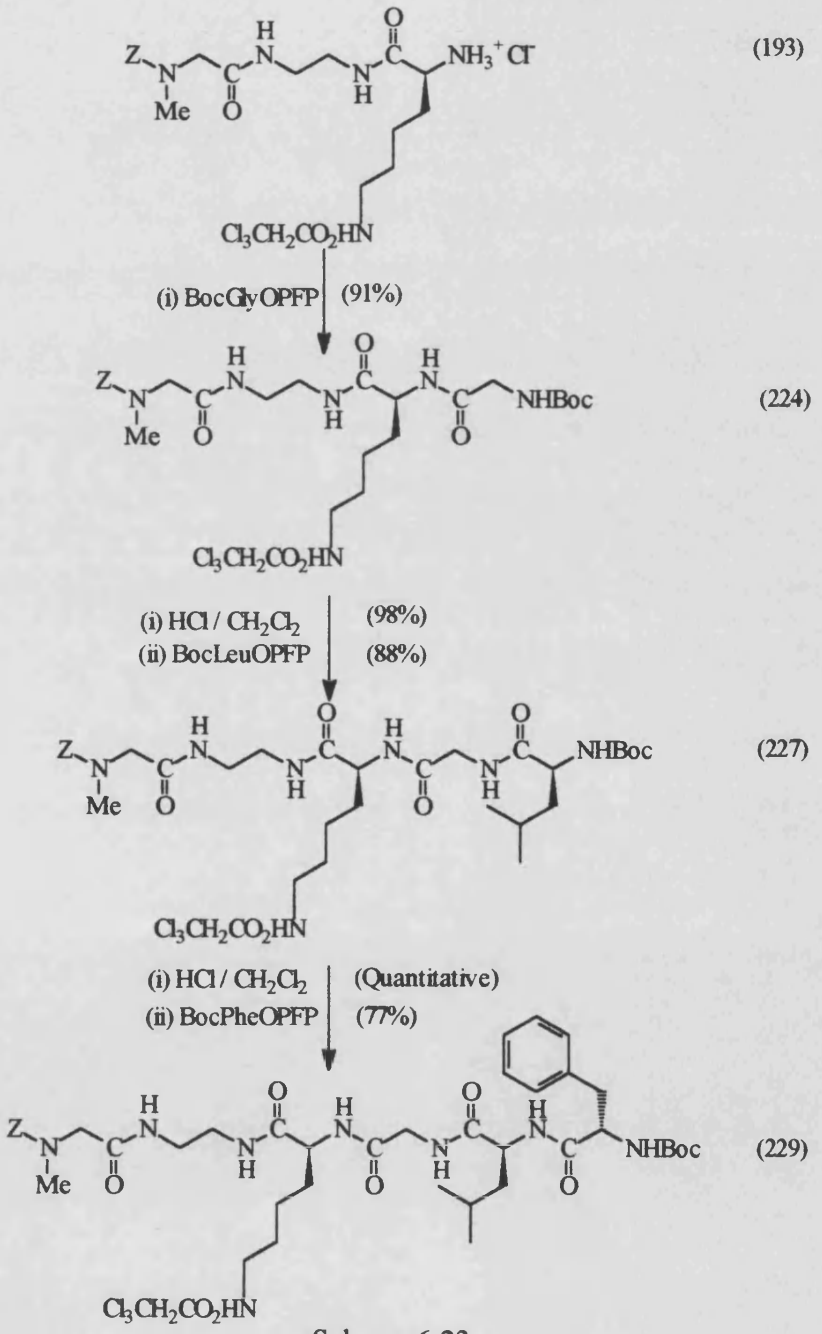
In an alternative approach, it was proposed to prepare monomers in which the derivatised amino acid and the degradable sequence were in a different order in the sequence. That is, the derivatised amino acid is adjacent to the ZSar-ethane-1,2-diamine unit. Initially, the glutamic acid monomer was investigated. As discussed earlier, the glutamic acid derivative can be easily coupled to the ZSar-ethane-1,2-diamine unit. BocGly can then be coupled to the deprotected compound. This coupling progressed very slowly with the reactants coming out of solution. However,

the desired compound was isolated in 74% yield on trituration with ether (Scheme 6.22). This was successfully deprotected by treatment with HCl in a mixture of THF and MeOH. Attempts to couple leucine proved unsuccessful, in that the reaction mixture gelled, requiring an increased dilution of the reactants and addition of further molar equivalents of the active ester. Even so, after 10 days the reaction had not proceeded to completion and the desired product could not be isolated due to the lack of solubility of both this product and the starting peptide. Thus, it is apparent that inclusion of the glutamic acid derivative enables easy isolation of some products due to ease of precipitation, but can reduce the rate of coupling of further amino acids to the sequence. Later results, in which the coupling of a spacer to lysine monomers proved difficult due to low solubility suggest that the lack of solubility, is due to a property of the spacer unit rather than due to the simple glutamic acid structure.

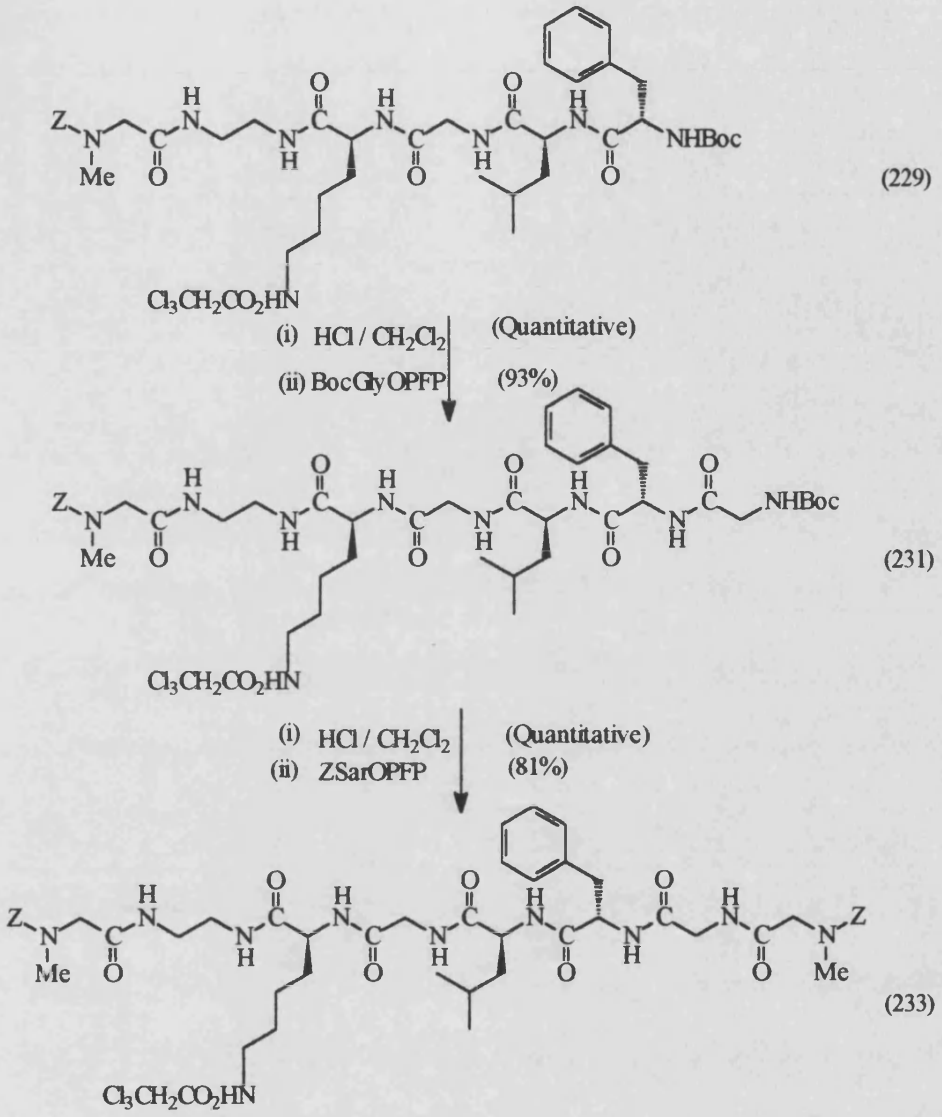



Scheme 6.22

Therefore, the peptide monomer with glutamic acid at the C-terminus was abandoned. However, the corresponding lysine monomer was easily prepared. In this case, all amino acids were coupled through their PFP esters to ensure efficient coupling. Also, in the latter stages of synthesis, as all products were to be isolated by column chromatography, an excess of the active amino acid was used to enhance the yield of the coupling reactions. Thus, **(233)** was easily prepared using standard methods.




Scheme 6.23

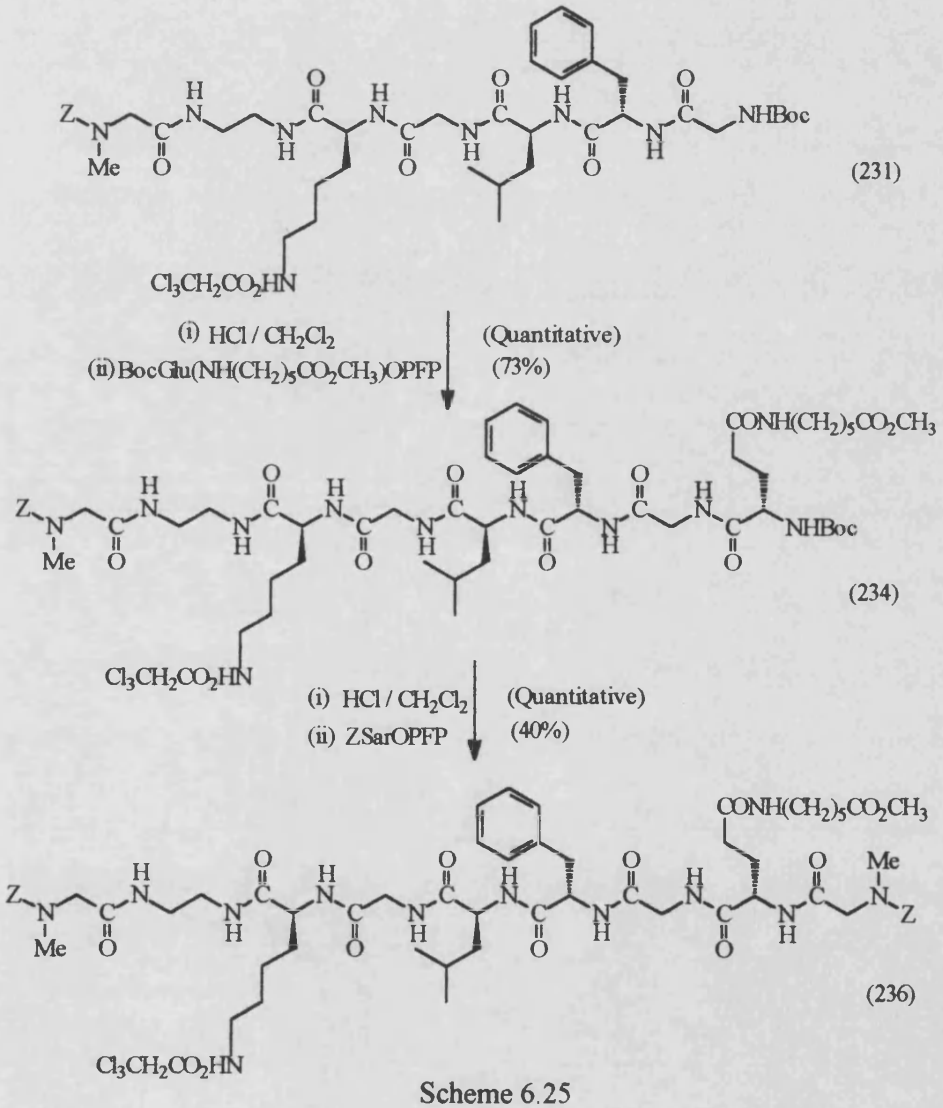



Scheme 6.24

6.4.2 Monomer incorporating two functionalised amino acids

To achieve attachment of the porphyrin, only one functionalised amino acid is required in the sequence. However, if a drug were attached to the monomer, it would be advantageous to have more than one functionalised amino acid in the sequence to achieve the highest loading possible. Also, if there was more than one functionalised amino acid, it would be possible to attach a targeting moiety as well as a drug. To

achieve a high loading, the same amino acid should be incorporated twice. To attach a targeting group, however, it is desirable to prepare a monomer which contains different functionalised amino acids, preferably with orthoganality of protecting groups. Towards this latter aim, a monomer containing both glutamic acid and lysine has been prepared. The glutamic acid derivative was easily coupled to **(232)** to give **(234)** in 73% yield. This compound was then deprotected and coupled to ZSarOPFP to give the bis functionalised monomer **(236)** (40%) (Scheme 6.25).




Scheme 6.25

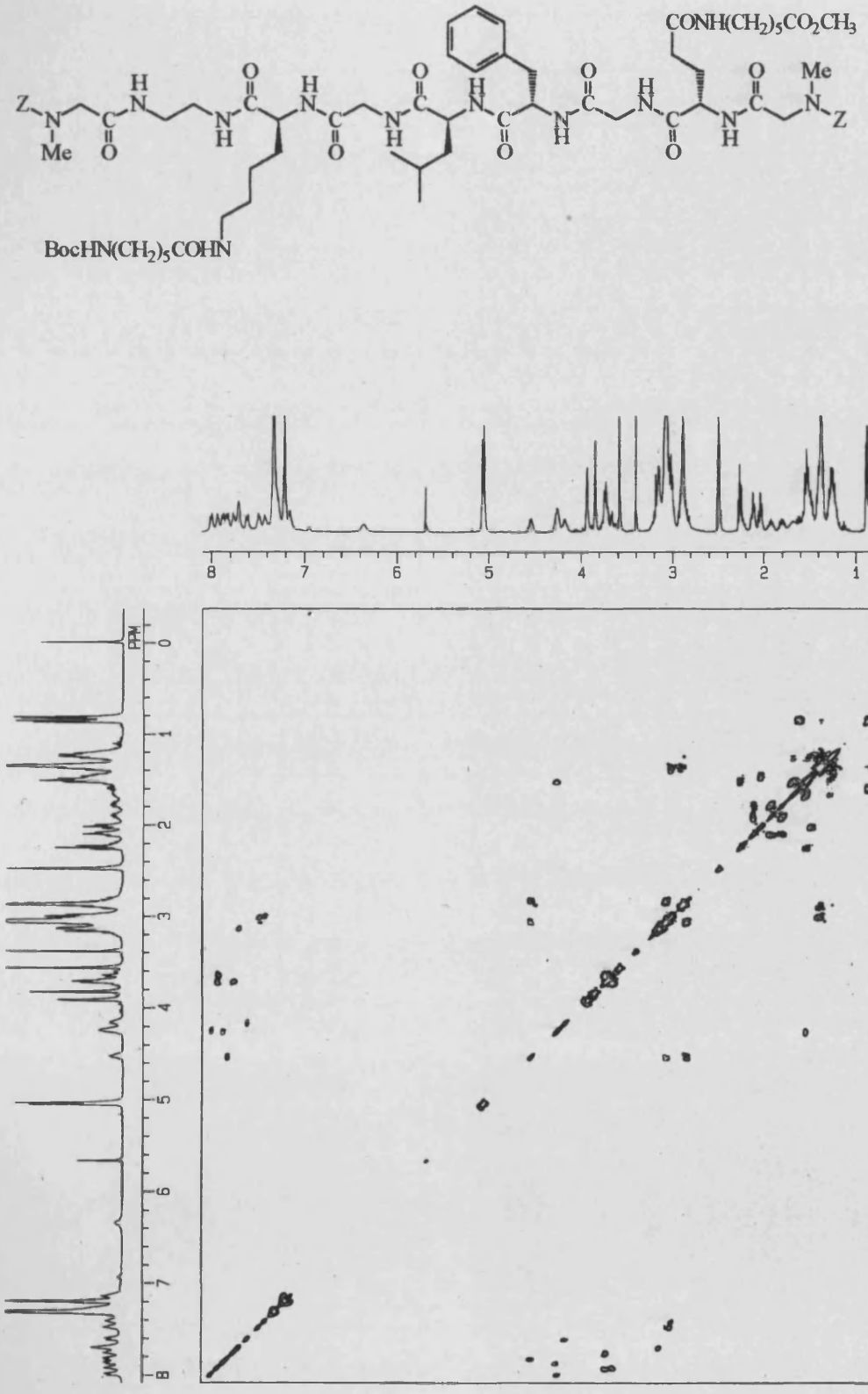
## 6.5 Characterisation of Peptide Derivatives

All peptides were characterised by proton NMR analysis. Appropriate intermediates were also characterised by $^{19}F$ NMR. Due to restricted rotation around the C-N bond in both Z-and Boc-protected sarcosines, all peptides containing this residue exhibited two sets of signals for both the Sar $CH_3$ and $CH_2$ and also for the benzyl $CH_2$ in the Z derivative. Heating the samples to 80°C confirms that these signals are due to rotamers as the signals coalesce to give a simpler spectrum. For all peptide sequences it was possible to characterise authoritatively the compounds. For the larger sequences, 2D COSY analysis was required to establish exact assignments of the spectra. An example of such a spectrum for compound **259** is shown on page 154.

In a normal spectrum the Leu-$CH_3$ is found between δ 0.7-0.9 and the $CH_2$ signals for Lys, Leu, Glu and the central portion of the spacer between δ 1.0-2.0. Glu γ-$H_2$ and the spacer $CH_2$ adjacent to a carboxyl give triplets at approximately δ 1.8-2.2. The Phe β-$H_2$ signals are seen as two separate double doublets at around δ 2.8 and δ 3.05. The ethane-1,2-diamine protons give a broad signal at δ 3.11. Lys ε-$CH_2$ and the spacer $CH_2$ adjacent to nitrogen appear as overlying multiplets. Gly $CH_2$, as a multiplet and Sar-$CH_2$, as a singlet, are located between δ 3.5-4.0. All the α-H give signals between δ 4.0-5.0. The $CH_2$ in both the Troc and Z protecting groups are seen as singlets between δ 4.8 and 5.2. Phe and Z aromatic protons give multiplets in the region δ 7.1-7.4. The signals due to NH protons are seen between δ 6.8 and 9.0.

The identification of all the peptide sequences was confirmed by FAB mass spectra. Certain key intermediates and target compounds were also subjected to accurate FAB mass spectral analysis.

Figure 6.6. COSY 90 : Compound **259** (80°C)



## 6.6 Conclusion

Using an orthogonal protecting group strategy based on Z and Boc protection and active ester coupling procedures, a number of peptide monomers have been prepared. Three non-degradable monomers, suitable for the attachment of a derivatised porphyrin and polymerisation, were initially synthesised. Subsequently, five monomers have been prepared incorporating the degradable unit GlyPheLeuGly. Four of these monomers also contain a side chain functionalised amino acid for porphyrin attachment.

# CHAPTER SEVEN

# PORPHYRIN-PEPTIDE CONJUGATES

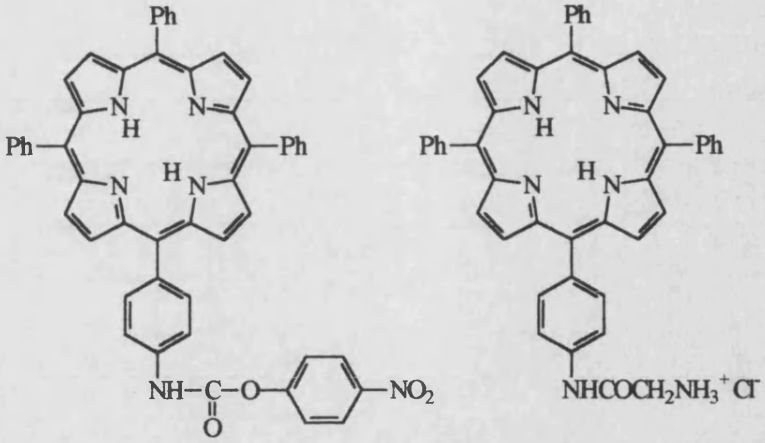
## 7.1 Introduction

As described in Chapters 5 and 6, both the porphyrin and peptide components of the polymer have been prepared. For ease of synthesis and characterisation, it is desirable to attach the porphyrin to the peptide monomer prior to polymerisation. This would also enable efficient loading of porphyrin on the polymer. Conjugation after polymerisation is likely to proceed slowly, owing to steric interference, and would be unlikely to result in quantitative loading.

However, with the monomer containing both a glutamic acid and a lysine residue, only one of the amino acids will be used for the coupling of the porphyrin derivative. The other amino acid will remain protected throughout the porphyrin coupling and polymerisation. After polymerisation, the protecting group could be removed and used to attach targeting groups, such as MSH. In the attachment of such groups, the loading ratio is of lesser importance as only one interaction between the polymer and cellular receptor is required to enable targeting. Thus, the porphyrin derivatives **(125)** and **(128)** (Figure 7.1) have been coupled to lysine and glutamic acid residues to give nine peptide-porphyrin conjugates suitable for polymerisation, following deprotection, with PEG.

## 7.2 Lysine-Porphyrin Monomers

In order to prepare a conjugate of a lysine monomer and the electrophilic porphyrin derivative **(125)**, it is first essential to remove the Troc protecting group to reveal the primary amine. This is suitable for direct coupling with the porphyrin. However, in a number of cases, a six-carbon chain spacer has been coupled to the peptide prior to




(125) (128)

Figure 7.1

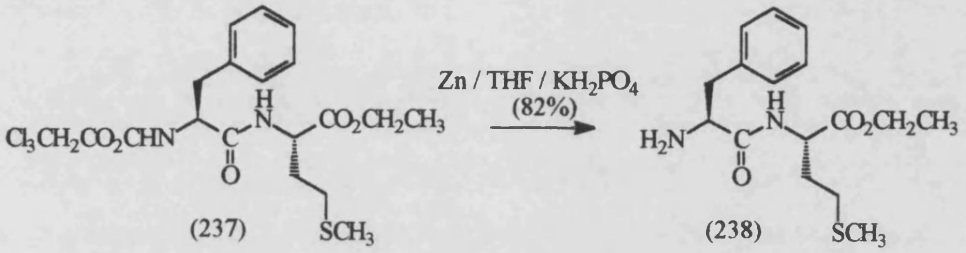
treatment with the activated porphyrin. This enables the investigation of two previously reported properties of polymers. Firstly, coupling of a porphyrin to a poly(amino acid) has been demonstrated to be increased if a spacer unit is incorporated into the polymer [215,216,217]. If this is the case, more efficient loading of the porphyrin could be expected to lead to an increased incorporation of manganese per macromolecule and thus to an increased relaxivity per mole of polymer. Secondly, it has been suggested that the relaxivity of a macromolecule is dependent on its rigidity [160]. Incorporation of a spacer unit would impart greater flexibility to the system. Thus it could be proposed that inclusion of the spacer would reduce the relaxivity of the macromolecule.

### 7.2.1 Removal of Troc

Removal of Troc is a relatively simple procedure. Most methods invoke a reductive mechanism in which zinc is used to abstract a chlorine atom from the protecting group forcing alkyl-oxygen fission. The most well known method involves treatment of the peptide with zinc in acetic acid, although numerous other solvents have been used [265,279]. The main problem with all these approaches is that some zinc salts are soluble in the solvent and thus are difficult to remove from the peptide. This could prove

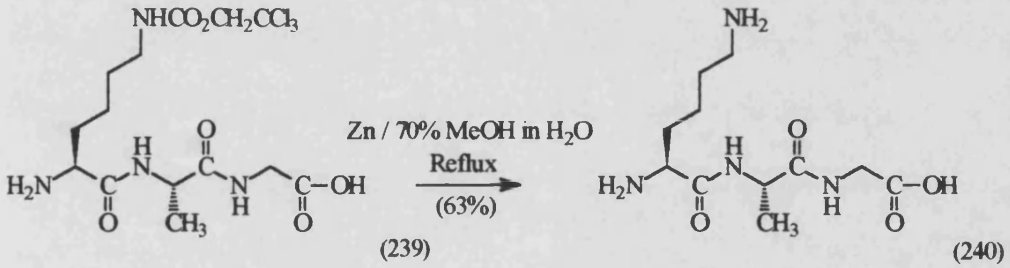
critical where a porphyrin is attached to a deprotected peptide as, if the peptide is not pure, zinc could be incorporated into the porphyrin.

An alternative method, which reduces the amount of zinc which is solubilised, was proposed by Just and Grozinger [279]. In this approach, the protected peptide is treated with zinc and acid ($KH_2PO_4$) in THF. With this procedure, high yields of around 80% are achieved (Scheme 7.1).



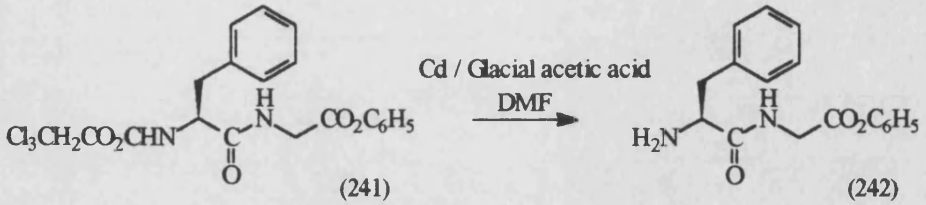

Scheme 7.1

Yajima *et al* [278] described a process whereby the peptide is treated with zinc in a refluxing methanolic solution. This is an attractive method as it is simple and provides the deprotected peptide in high yield (Scheme 7.2).




Scheme 7.2

In a comprehensive study on potential solvents for zinc-based reduction of the Troc group, Hancock *et al.* also demonstrated the ability of cadmium to afford peptide deprotections. Deprotection of **(241)** was achieved in eight hours by treatment with cadmium dust in a 1 : 1 mixture of acetic acid and DMF (Scheme 7.3).



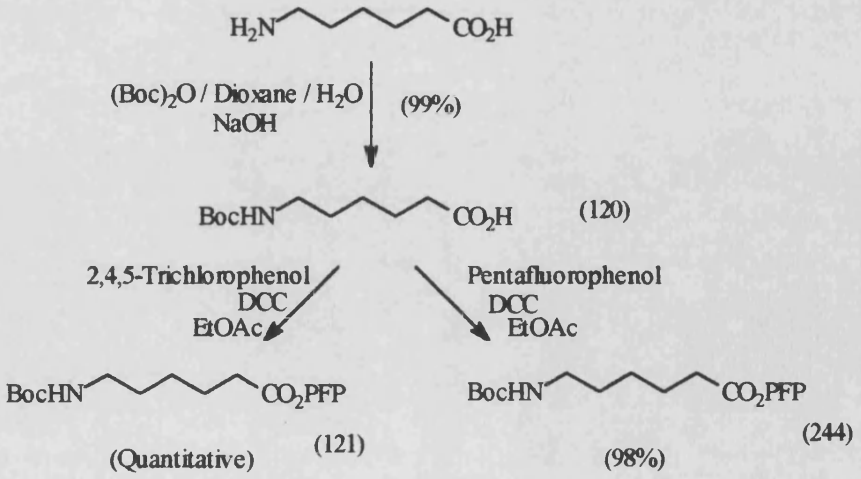

Scheme 7.3

7.2.2 Non-Degradable Monomers

Initial experiments were performed on the non-degradable monomer **(194)**, as a model for the more complex degradable monomers.

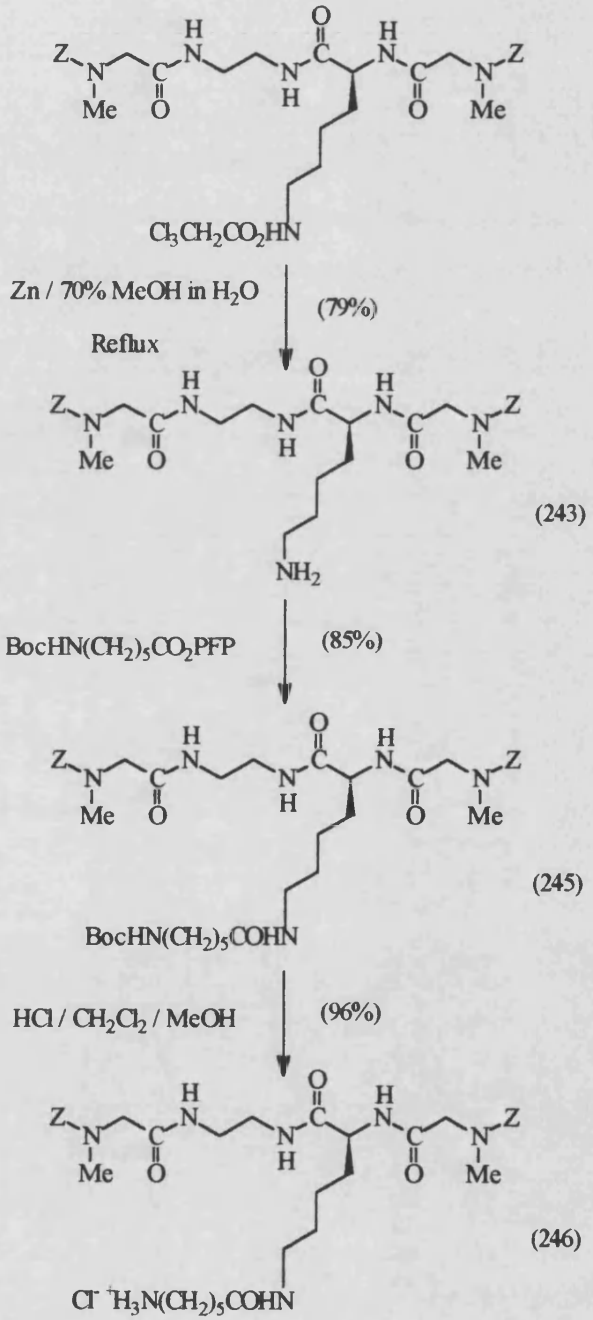
The mild conditions developed by Yajima [278] were employed in the deprotection of this monomer. Deprotection was highly successful, in that the free amine was isolated in 79% yield after 7 hours treatment with zinc in 70% methanol in water. In a slight modification of this method, the product **(243)** was isolated by evaporation of the solvent mixture following filtration.

With this monomer, a spacer was then incorporated. The spacer chosen was based upon 6-aminohexanoic acid. This was easily protected with Boc and then activated as the pentafluorophenyl ester (Scheme 7.4).



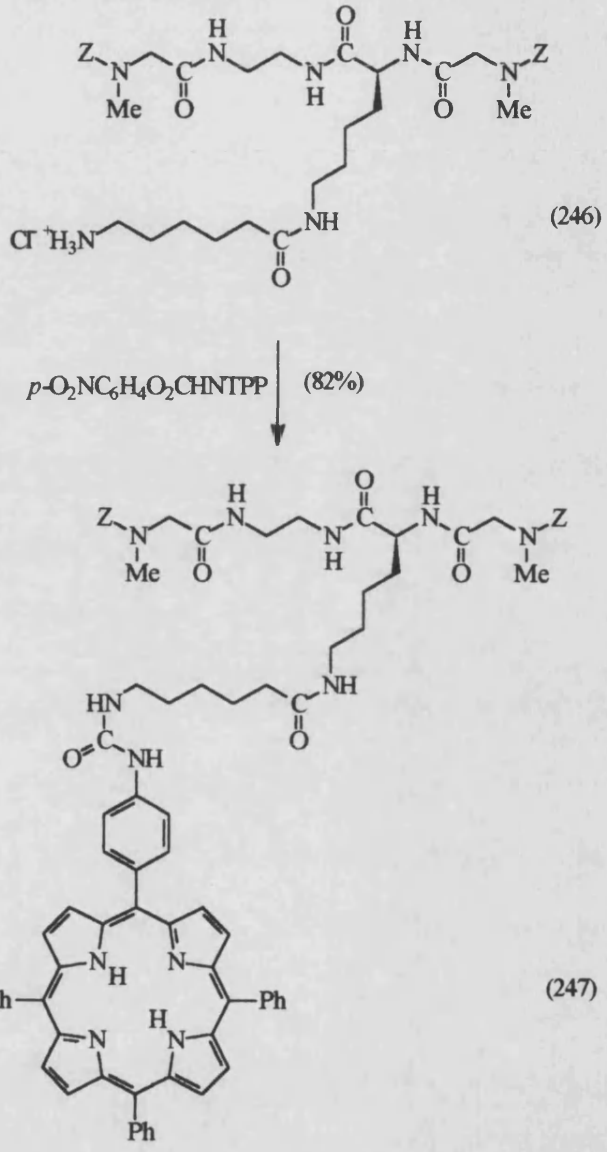

Scheme 7.4

Coupling of the spacer to the peptide initially proved problematic. Gelling of the reaction mixture was observed, similar to the gelling seen previously with the glutamic acid monomers (Chapter 6). However, this problem was overcome by using a mixture of solvents, $CH_2Cl_2$ and DMF, and an excess of the activated spacer. Thus **(245)** was isolated, following chromatography, in 85% yield.




Scheme 7.5

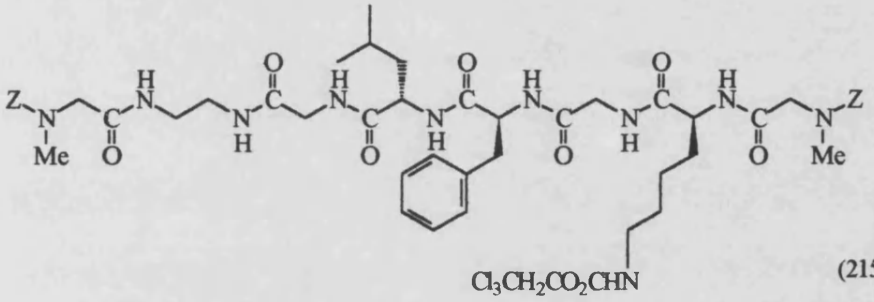
This compound was easily deprotected by treatment with HCl to provide an amine for coupling with the activated porphyrin **(125)**. Again to ensure solution, this reaction was performed in a mixed solvent system, $CH_2Cl_2$ / MeOH. Coupling with the porphyrin was achieved in 24 hours giving the peptide-porphyrin **(247)** in 82% yield.



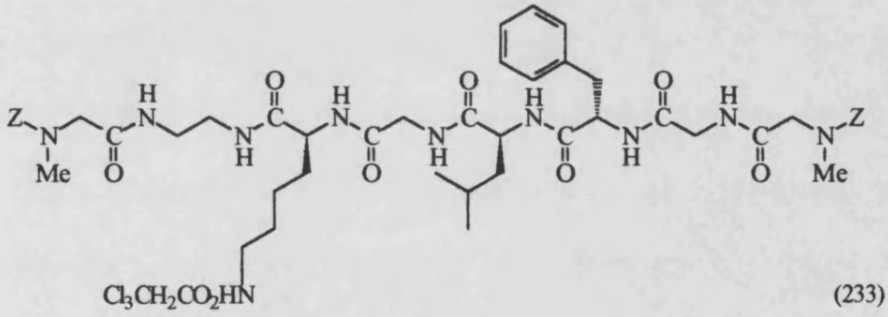

Scheme 7.6

7.2.3. Degradable Monomers

Thus the synthetic strategies for the deprotection, incorporation of spacer and coupling of porphyrin have been successfully developed. This approach was then used to prepare the porphyrin conjugates of both peptide monomers consisting of lysine together with the degradable unit **(215)** and **(233)** (Figure 7.2). With both these monomers, two conjugates were prepared, one in which the porphyrin is directly linked to the lysine residue and one employing a spacer between the porphyrin and peptide.




(215)

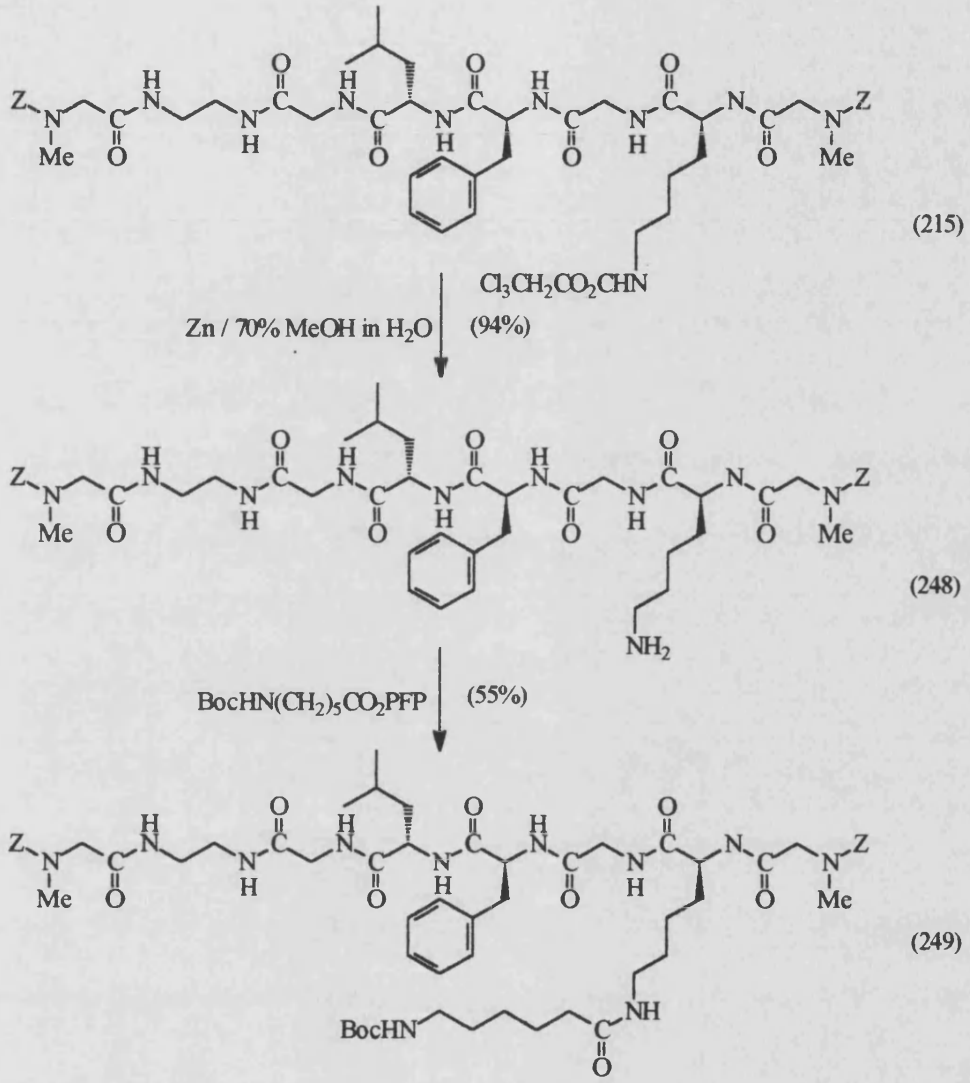



(233)

Figure 7.2

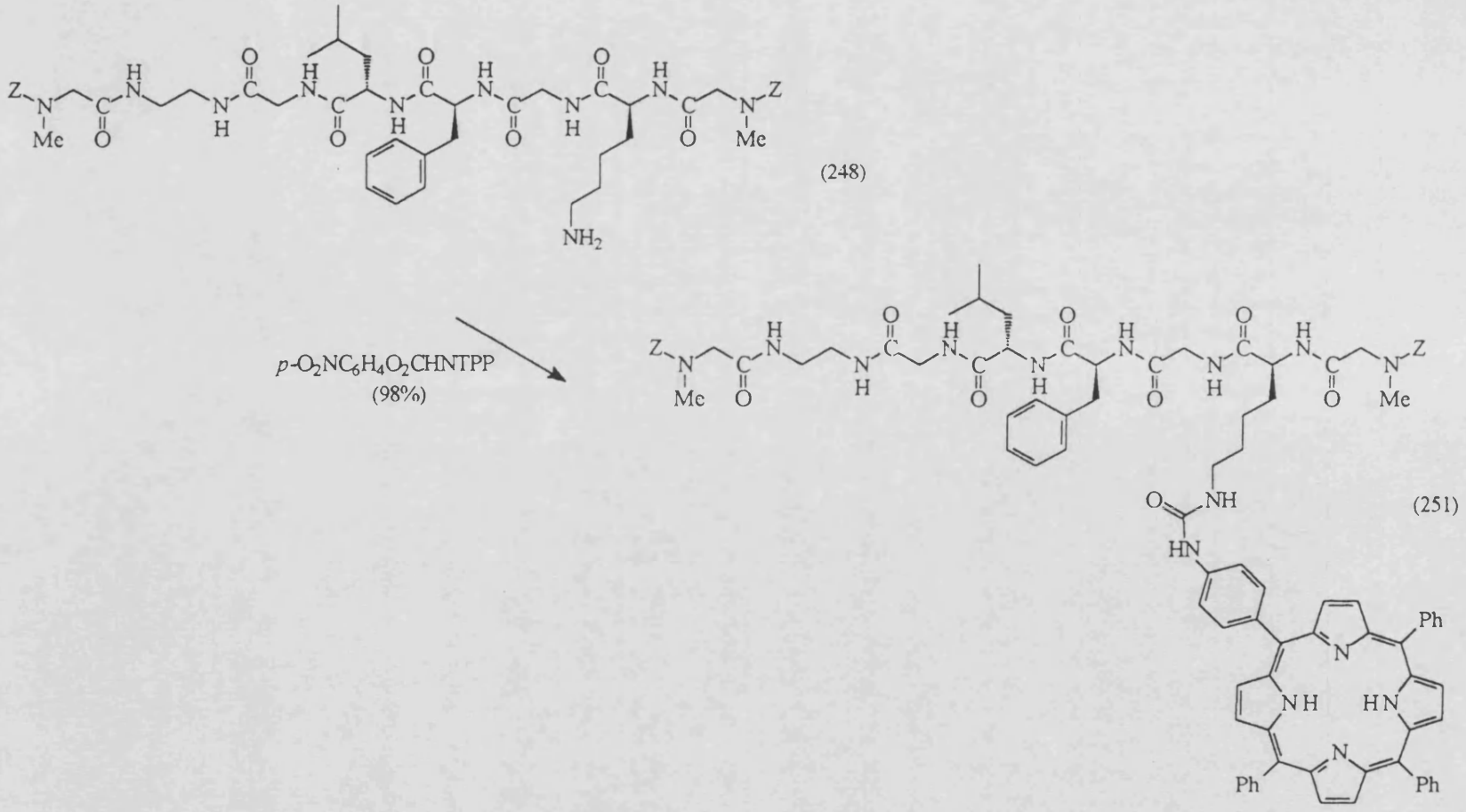
With monomer **(215)**, removal of the Troc protecting group with zinc in methanol was successfully achieved (94%). However, prolonged heating was required, complete conversion only being accomplished after 24 hours. Coupling of the spacer to this compound again proved problematic. Despite use of a great excess of spacer and a mixed solvent system, coupling proceeded slowly and incompletely. Thus, the

extended peptide **(249)** was prepared in 55% yield. This product was, as expected, of low solubility but was easily deprotected to provide a primary amine for coupling to the porphyrin.




Scheme 7.7

Porphyrin conjugates were then prepared. With the non extended monomer **(248)**, the coupling was carried out in a DMF and $CHCl_3$ solution to obviate potential problems of gelling of the reaction mixture as seen with the coupling of spacers. Chromatography of the reaction mixture gave the peptide-porphyrin conjugate **(251)** in 98 % yield (Scheme 7.8).

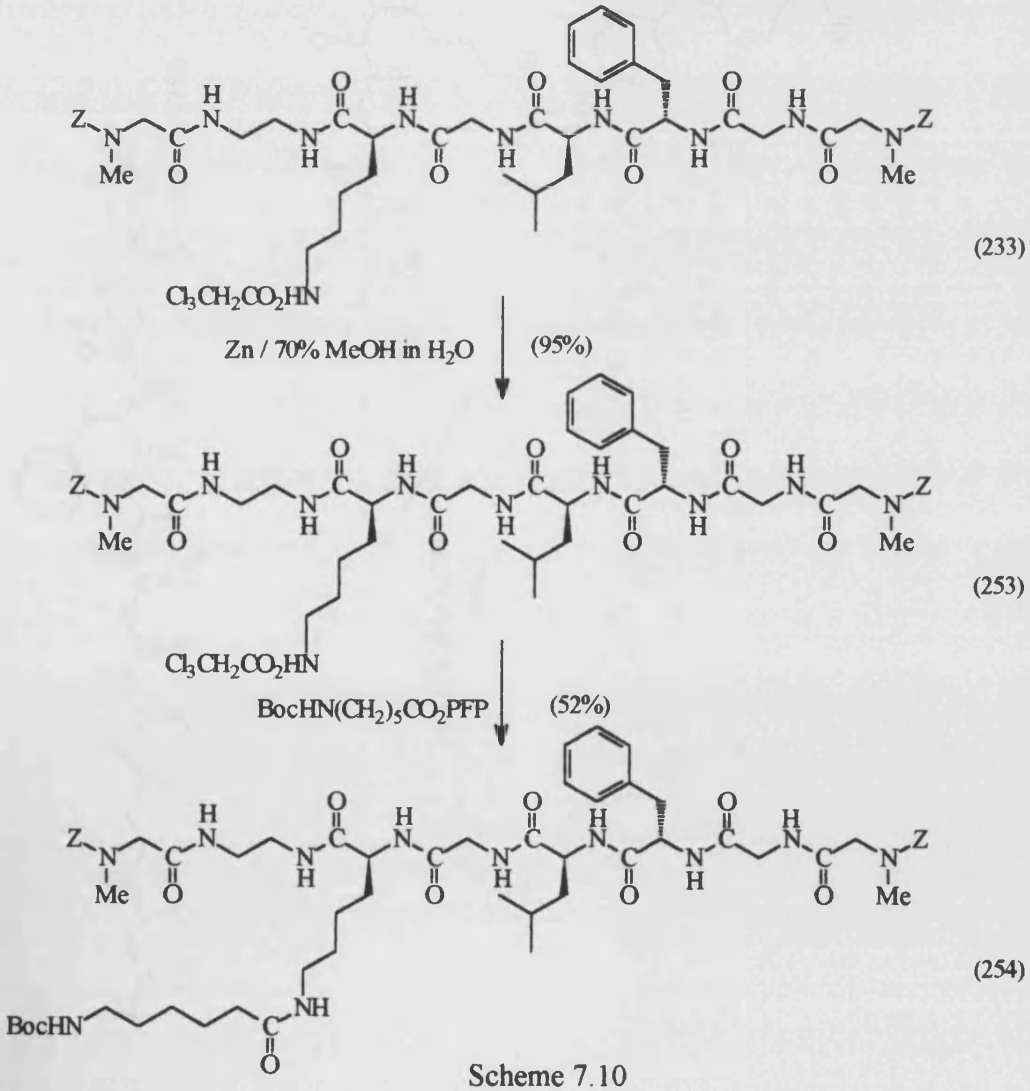



Scheme 7.8

Scheme 7.9

As it was of known low solubility, a mixed solvent system was again employed with the extended monomer. This coupling was less successful than with the corresponding non-extended monomer. Complete conversion of the activated porphyrin was achieved within 24 hours, but more than one product was formed. Thus, **(252)** was isolated in 64% yield.

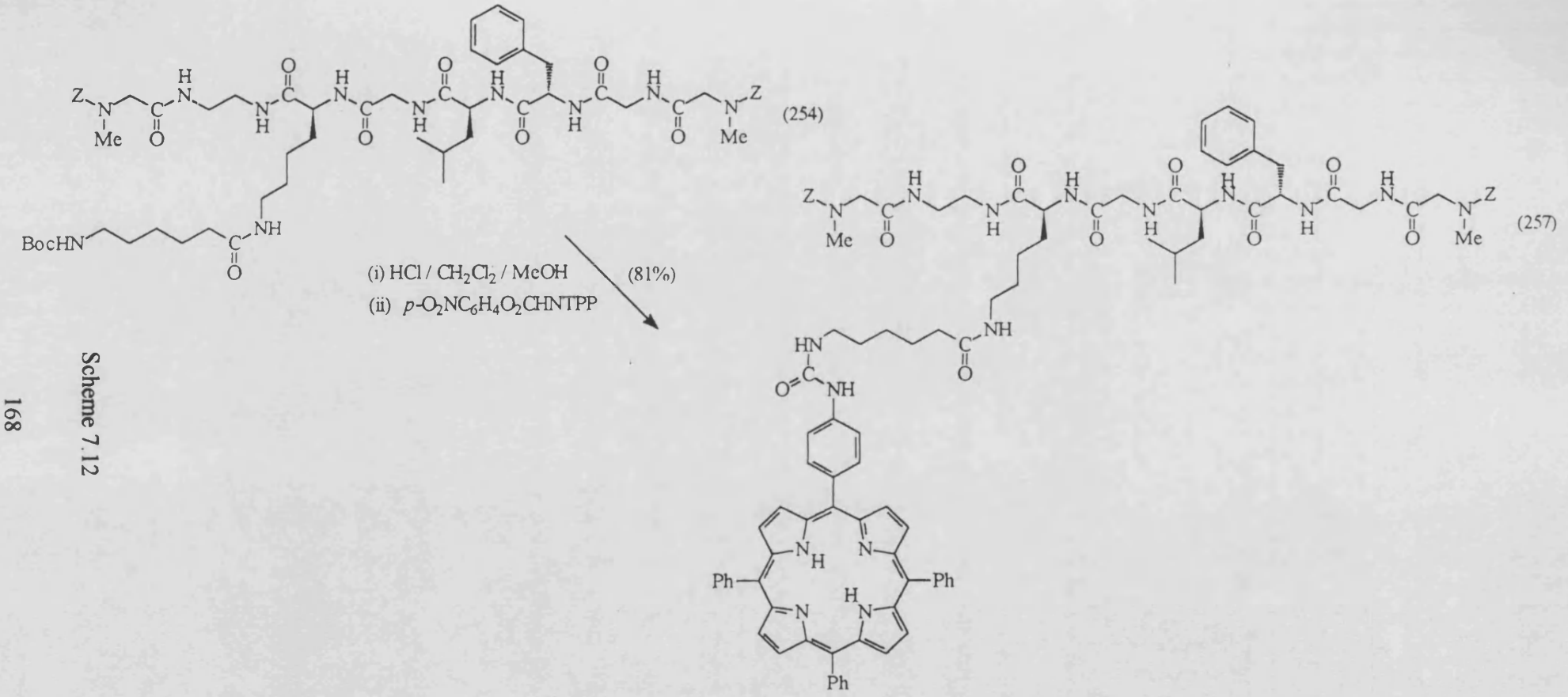
Similar problems were encountered with the synthesis of the porphyrin conjugates of monomer **(233)**. With this monomer however, cleaner coupling of the porphyrin to the extended monomer was achieved. Thus **(257)** was prepared in 81% yield.




Scheme 7.10

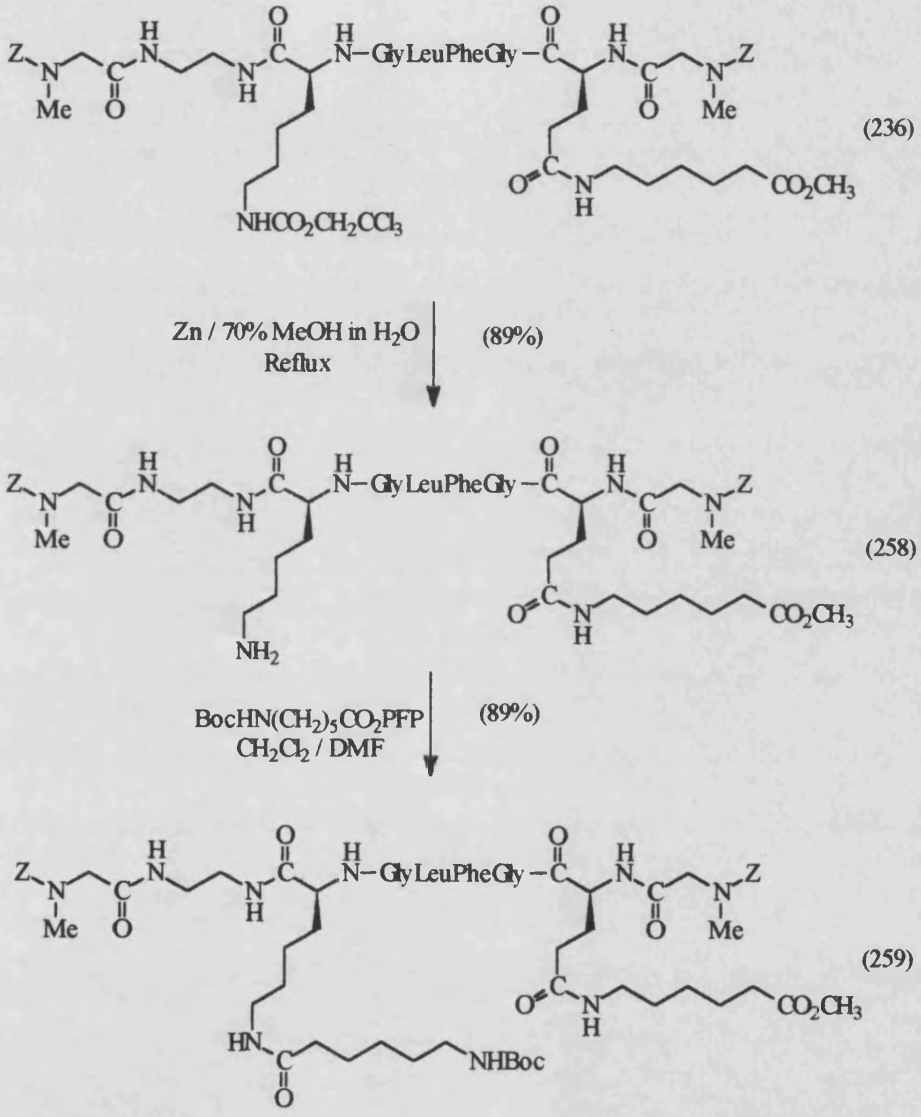
Z
N
Me
O
H
N
O
H
N
O
H
N
O
H
N
O
H
N
O
H
N
O
N
Me
Z
NH2
(253)
p-O2NC6H4O2CHNTPP
(84%)
Z
N
Me
O
H
N
O
H
N
O
H
N
O
H
N
O
H
N
O
N
Me
Z
(256)
O
NH
HN
Ph
N
NH
HN
N
Ph
Ph

Scheme 7.11




Scheme 7.12

In a final study, the monomer containing both functionalised amino acids (Glu and Lys) was coupled to a porphyrin through the lysine residue. Obviously, in this case, deprotection of the lysine residue must be achieved without a loss of integrity of the glutamic acid protection. Deprotection of the monomer was achieved using zinc in 70% methanol in water without loss of the methyl ester. Coupling of the spacer, as its active ester, was highly efficient giving **(259)** in 89% yield.




Scheme 7.13

As this product was expected to be only slightly soluble in $CH_2Cl_2$, this coupling was again performed in a mixed solvent system. The base used in the reaction was also

altered, to the more volatile diethylmethylamine, to enable efficient isolation of the product by chromatography. This may, however, have also been responsible for the increased efficiency of the coupling reaction. The Boc protecting group was easily removed. Coupling of the porphyrin **(125)** gave a number of products from which **(261)** was isolated in 85% yield.

Thus, the attachment of a monofunctionalised porphyrin to a peptide monomer containing lysine has been achieved in high yield in a variety of situations. Inclusion of the spacer has, if any, a negative effect on the coupling of the porphyrin, in contrast to previously reported results. It is yet to be seen if inclusion of the spacer has any effect on proton relaxation enhancement.

## 7.3 Glutamic Acid-Porphyrin Monomers

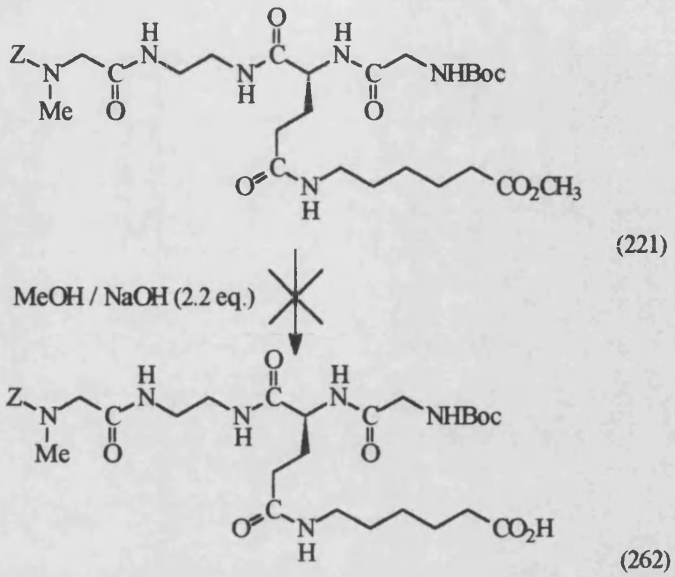
### 7.3.1 Non-Degradable Monomers

As has been described in chapter 6, three monomers containing an extended glutamic acid residue have been prepared. The carboxylic acid function in these monomers is protected as the methyl ester. Therefore, this protecting group must first be removed and then the peptide monomer activated to enable coupling of the monofunctionalised porphyrin **(128)**.

In a first approach, saponification was investigated as a method of ester removal. Following removal of the ester, it was intended to form a pentafluorophenyl active ester of the peptide monomer which could be coupled to the porphyrin using the previously developed methods.

Initial studies were carried out on protected peptide intermediates. Treatment of **(221)** with two molar equivalents of NaOH in MeOH gave, on acidification, a number of products, suggesting cleavage of the peptide.

Scheme 7.14

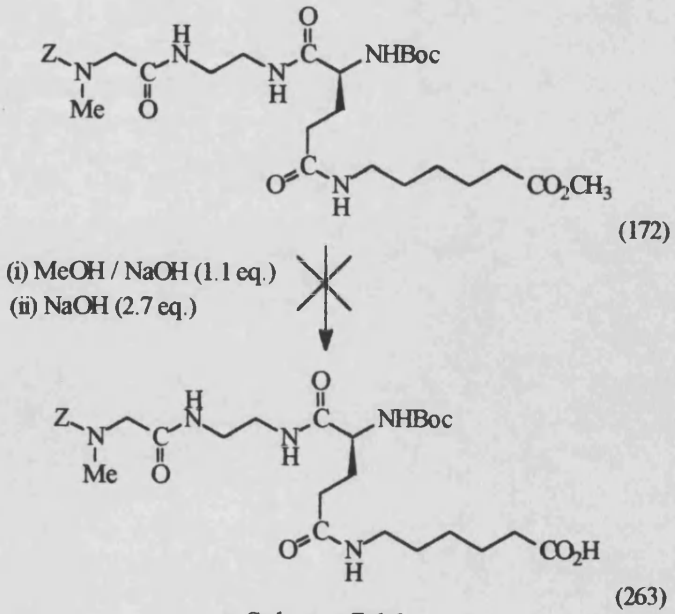



(221)

(262)

Scheme 7.15

In a second approach, less base was initially used in an attempt to avoid peptide cleavage. However, this reaction proceeded slowly, requiring further addition of base to achieve conversion of starting material. Again a number of products were formed. Thus it seems unlikely that selective cleavage of the methyl esters by base hydrolysis can be achieved without loss of the starting peptide.



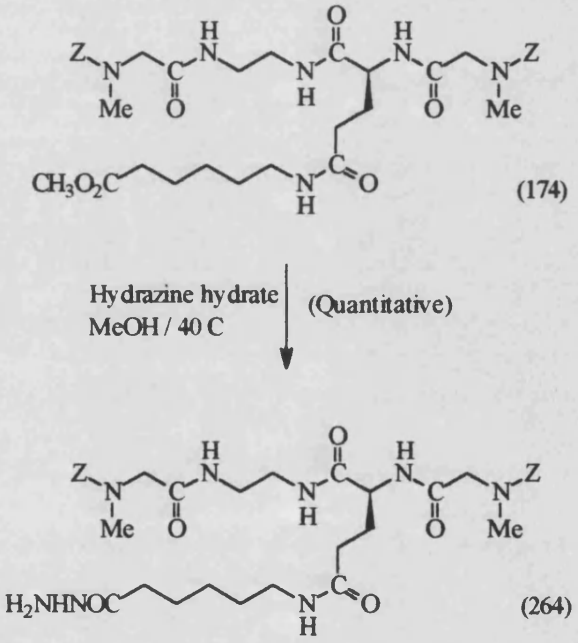

(172)

(263)

Scheme 7.16

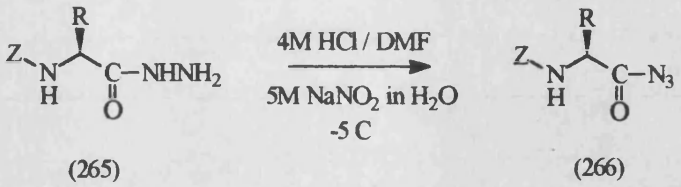
In an alternative approach, direct activation of the peptide was investigated. Hydrazides can be prepared from the esters of amino acids. These can then be activated to azides which can couple efficiently with amines. Thus the peptide could be converted to its hydrazide and coupled to the porphyrin via an azide.

The activation of the non degradable monomer **(174)** was first evaluated. Hydrazides are easily formed on treatment of an ester with an excess of hydrazine hydrate in methanol or ethanol [281,282,283]. Although, with a number of peptides, hydrazides can be formed at room temperature, for this monomer, the hydrazide was more efficiently prepared on heating to 40°C. Thus quantitative conversion was achieved in seven hours.




Scheme 7.17

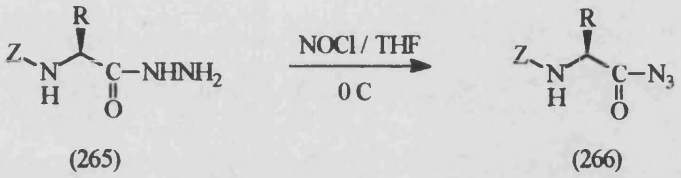
The formation of an azide from a hydrazide is more complex. Side reactions can occur leading to loss of activity or the formation of alternative products. In the majority of cases, the azide formed in a reaction is not isolated and is used directly to couple to an amine. A method which has been widely used in peptide synthesis involves the treatment of a hydrazide with an acidic solution of sodium nitrite (nitrous acid) [267] (Scheme 7.18 )



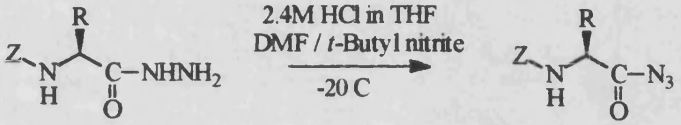

(265) (266)

Scheme 7.18

In a modification of this approach, Honzl and Rudinger [284] prepared azides using either butyl nitrite or nitrosyl chloride. Both of these methods are highly successful, however, at present, the butyl nitrite method has been most widely adopted [281] .



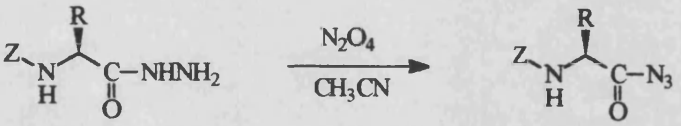

(265) (266)




(265) (266)

Scheme 7.19

Recently, two different methods have been presented in which azide formation is achieved through the action of nitrosonium ions on a hydrazide. Dinitrogen tetroxide is in equilibrium with nitrosonium ions in solution. the treatment of a hydrazide with dinitrogen tetroxide in acetonitrile results in the formation of an azide [285].




(265) (266)

Scheme 7.20

In a mechanistically related approach, Clayfen (clay supported ferric nitrate) has been used as a source of nitrosonium ions in the preparation of azides [286].

Side reactions are, however, a problem with azide formation. Inactive amides can be formed during azide preparation. This is probably due to rearrangement of an intermediate [283] rather than hydrolysis of the azide. Alternatively, azides, once formed, can undergo a Curtius rearrangement to the isocyanate. Both of these reactions lead to a reduced yield of the desired product in coupling reactions. However, reducing the temperature during azide formation can substantially reduce formation of these unwanted derivatives.

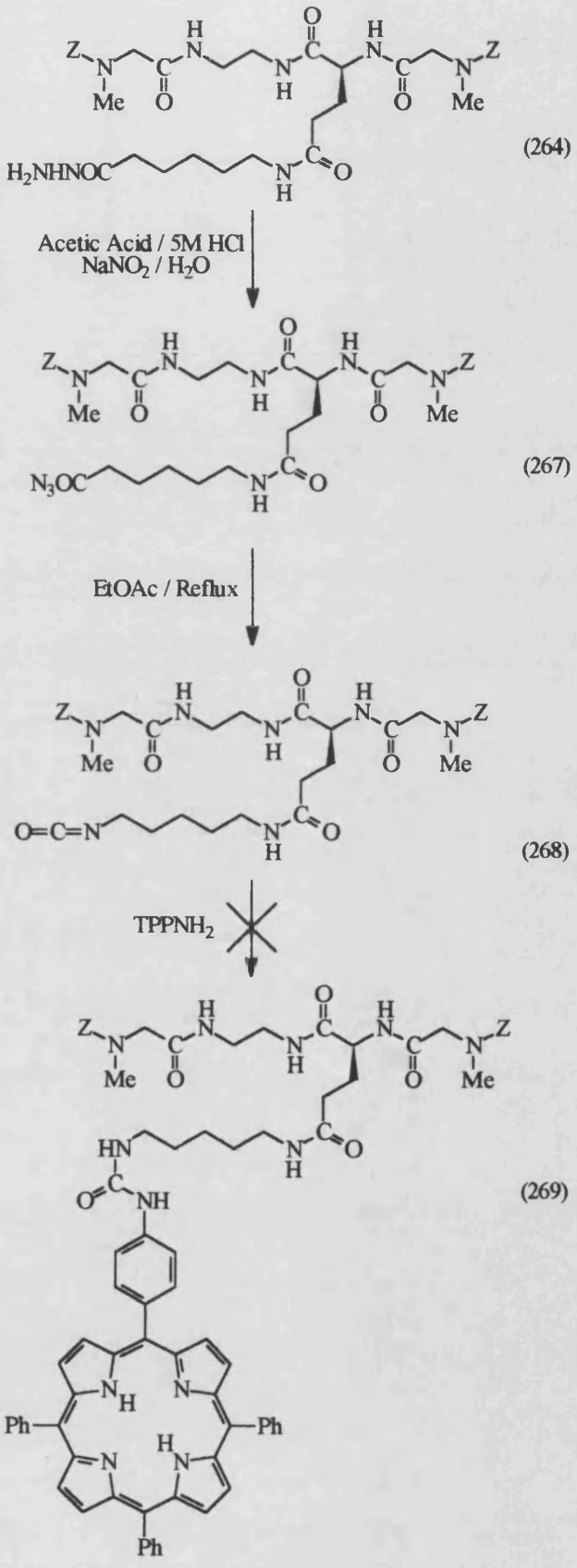
Although formation of the isocyanate is a problem in peptide synthesis, as peptide-like molecules linked by urea bonds are formed, it can be beneficial in some situations. Thus, some workers have prepared an azide and converted it to the more reactive isocyanate to afford the attachment of an antibody to a peptide sequence [282]. In this situation, the nature of the bond is not of importance. As has already been discussed, the porphyrin is linked to the lysine based monomers through a urea. Thus, it would be acceptable to have the glutamic acid linked to the porphyrin through a urea. When considering such an approach, it is essential to ensure complete conversion of the azide to the isocyanate. if this is not achieved a mixture of two products, with very similar physical properties, is likely to be formed during coupling making separation difficult.

Therefore in an initial approach to coupling the porphyrin to the small monomer **(174)**, the hydrazide **(264)** was converted to an azide **(267)** and then heated to force the Curtius rearrangement to the isocyanate. The hydrazide was converted to the azide using a nitrous acid method. The product was not isolated and was heated at reflux, in EtOAc, for 30 minutes to form the isocyanate. The reaction mixture was then treated with the porphyrin derivative **(93)**. A number of products were formed, none of which was the desired conjugate

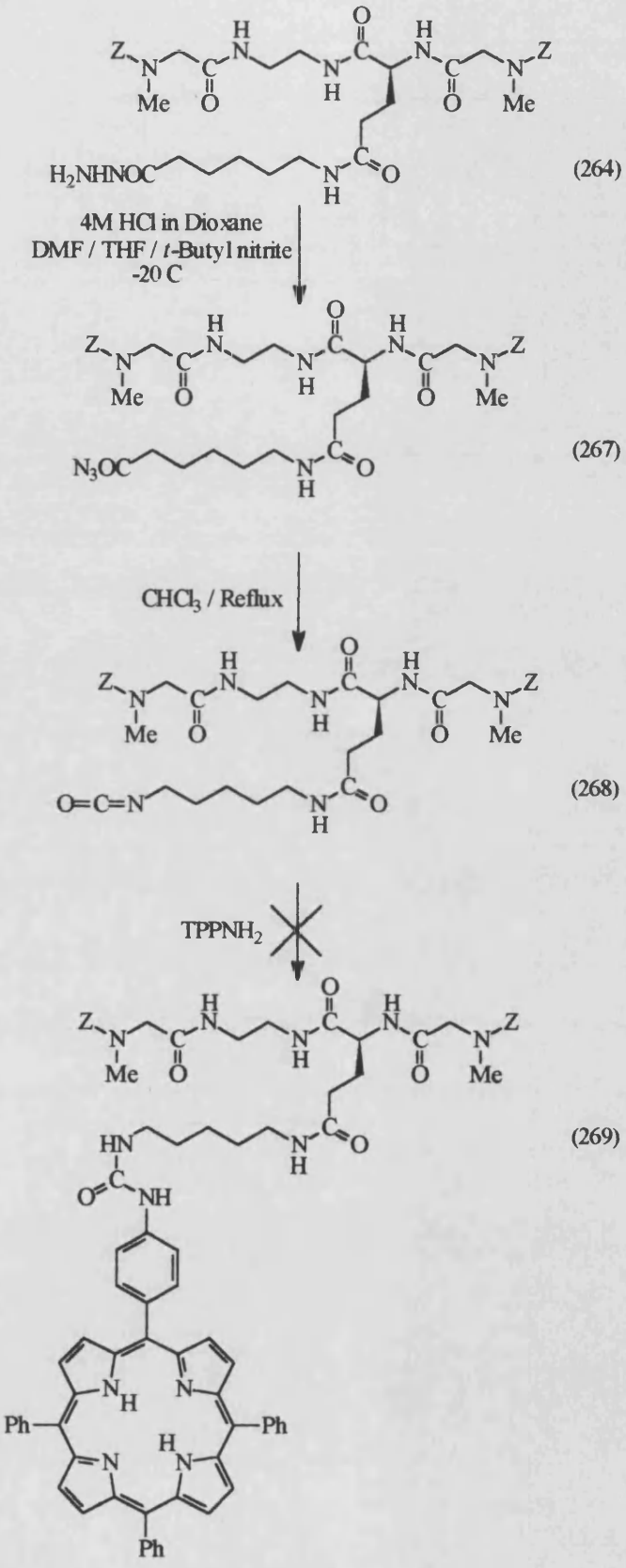
In a second attempt, the azide was prepared using a modification of the alkyl nitrite method of Tomatis *et al* [281]. The intermediate was heated in $CHCl_3$ and then treated with the porphyrin. Again a number of products were prepared. From these results it appears that other rearrangements take place on heating the azide, possibly to the amide, resulting in inactivation.

Thus the azide approach was re-evaluated. If the temperature is kept low, for both the activation and coupling reactions, isocyanate and amide formation should be minimal. Therefore, it should be possible to prepare a conjugate linked through a peptide bond at low temperatures. This was investigated using monomer **(174)**. The azide was formed by treatment of the hydrazide **(264)** with *tert*-butyl nitrite. This was then allowed to react with aminoporphyrin **(93)**. The porphyrin was added to the reaction mixture at -50°C and the vessel allowed to warm slowly to room temperature. After 24 hours coupling had been achieved giving **(270)** in 37% yield

This confirmed the suitability of the azide coupling reaction, however, the yield was disappointingly low. This was thought to be due to a lack of nucleophilicity of the aminoporphyrin rather than incomplete formation of the azide. This theory was confirmed by the reaction of the azide **(267)** with a more nucleophilic derivative of the porphyrin **(128)**. Coupling, using the same conditions, gave **(271)** in 76% yield.

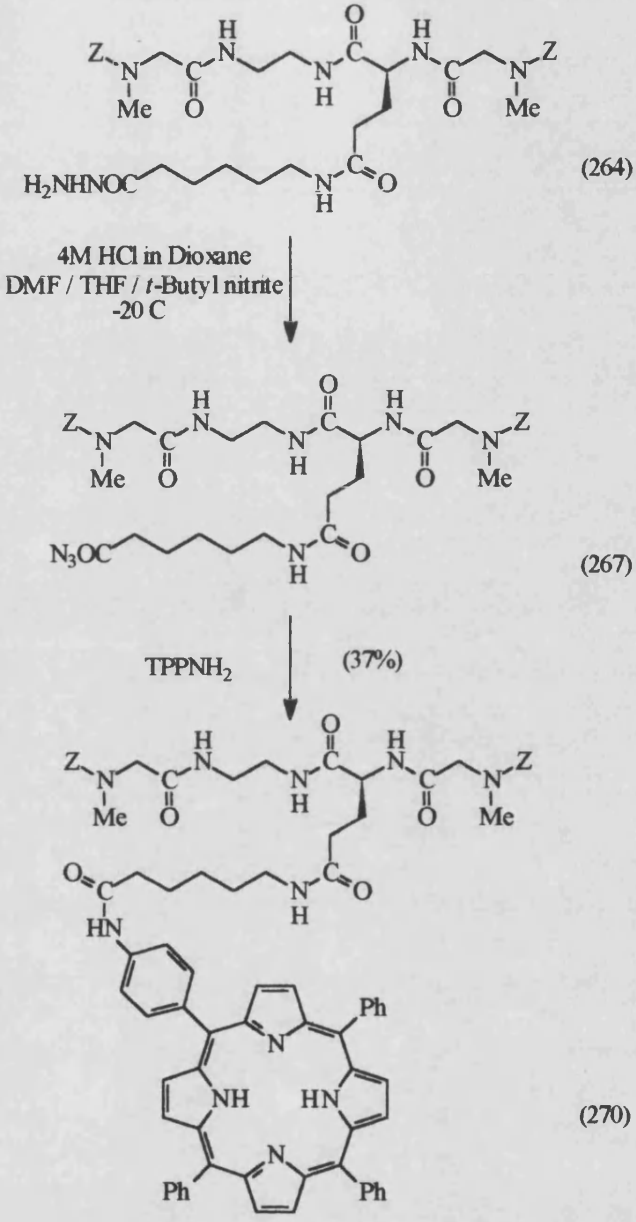

(264)
Acetic Acid / 5M HCl
NaNO2 / H2O
(267)
EtOAc / Reflux
(268)
TPPNH2
(269)

Scheme 7.21

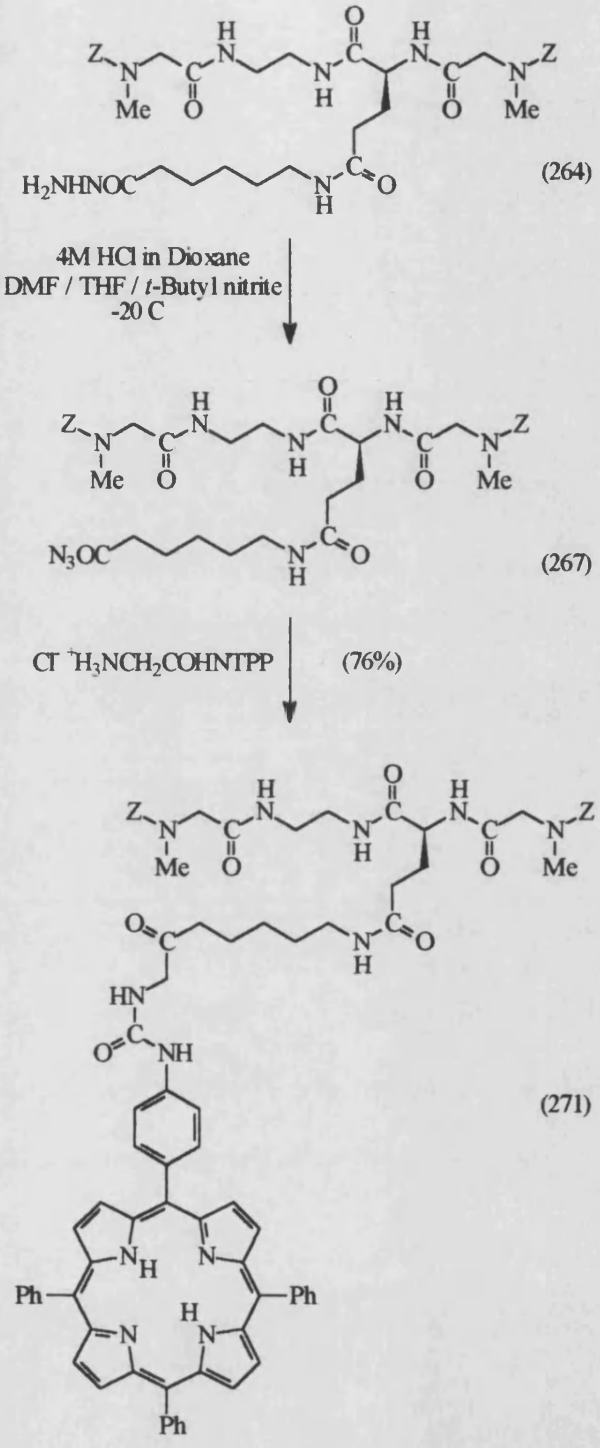



(264) (267) (268) (269)

Scheme 7.22


(264)
4M HCl in Dioxane
DMF / THF / t-Butyl nitrite
-20 C
(267)
TPPNH2
(37%)
(270)

Scheme 7.23

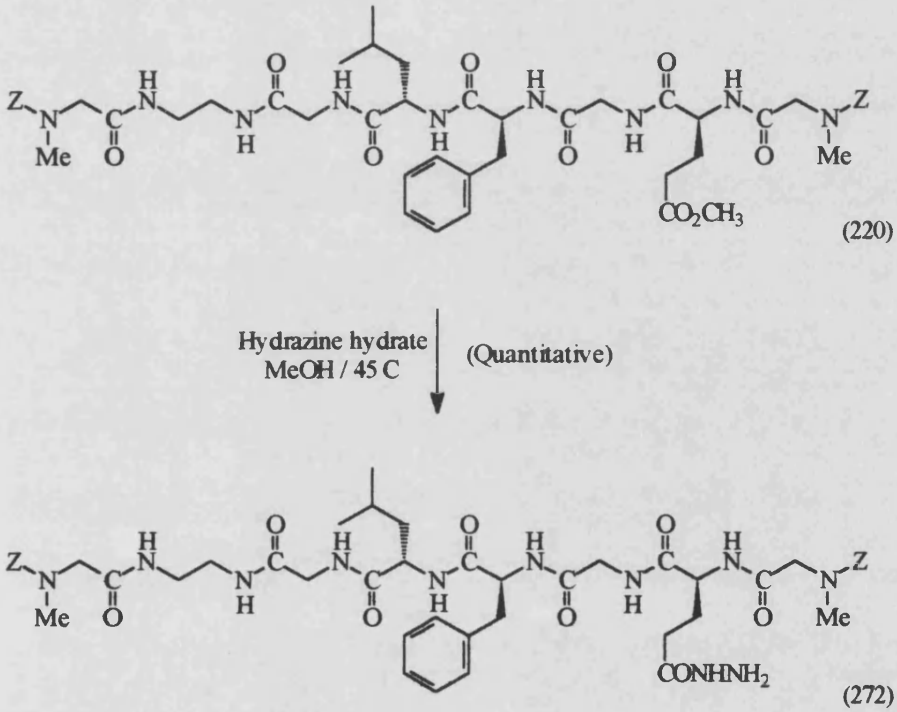

(264)
4M HCl in Dioxane
DMF / THF / t-Butyl nitrite
-20 C
(267)
Cl⁻ ⁺H3NCH2COHNTPP
(76%)
(271)

Scheme 7.24

7.3.2 Degradable Monomers

This success led to the extension of the azide coupling strategy, combined with activation of the porphyrin, to the degradable monomers containing glutamic acid **(220)** and **(233)**.

With the monomer consisting of only glutamic acid and the degradable sequence **(220)**, formation of the hydrazide proved difficult. It was necessary to heat the peptide in solution at 45°C for 48 hours to achieve complete conversion.




(220)

(272)

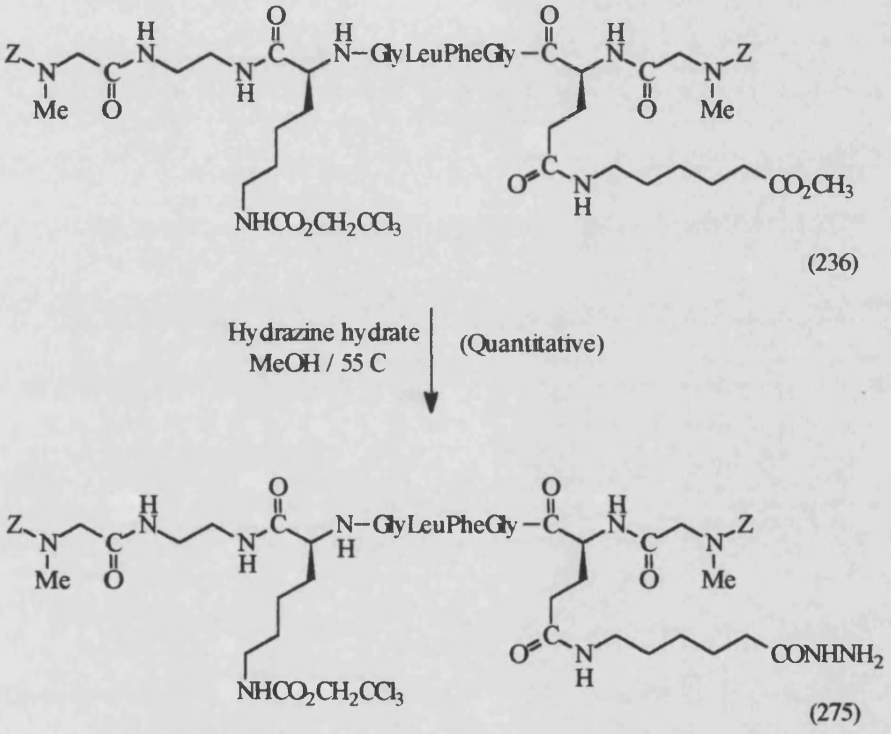
Scheme 7.25

This was then converted to the azide, again this required longer treatment with the nitrite. Coupling of the porphyrin, however was efficient giving **(274)** (83%) in 48 hours.

(272)
(i) 4N HCl in dioxane
DMF / THF/ t-Butyl nitrite
-20 C
(ii) Cl⁻ ⁺H3NCH2COHNTPP
(83%)
(274)

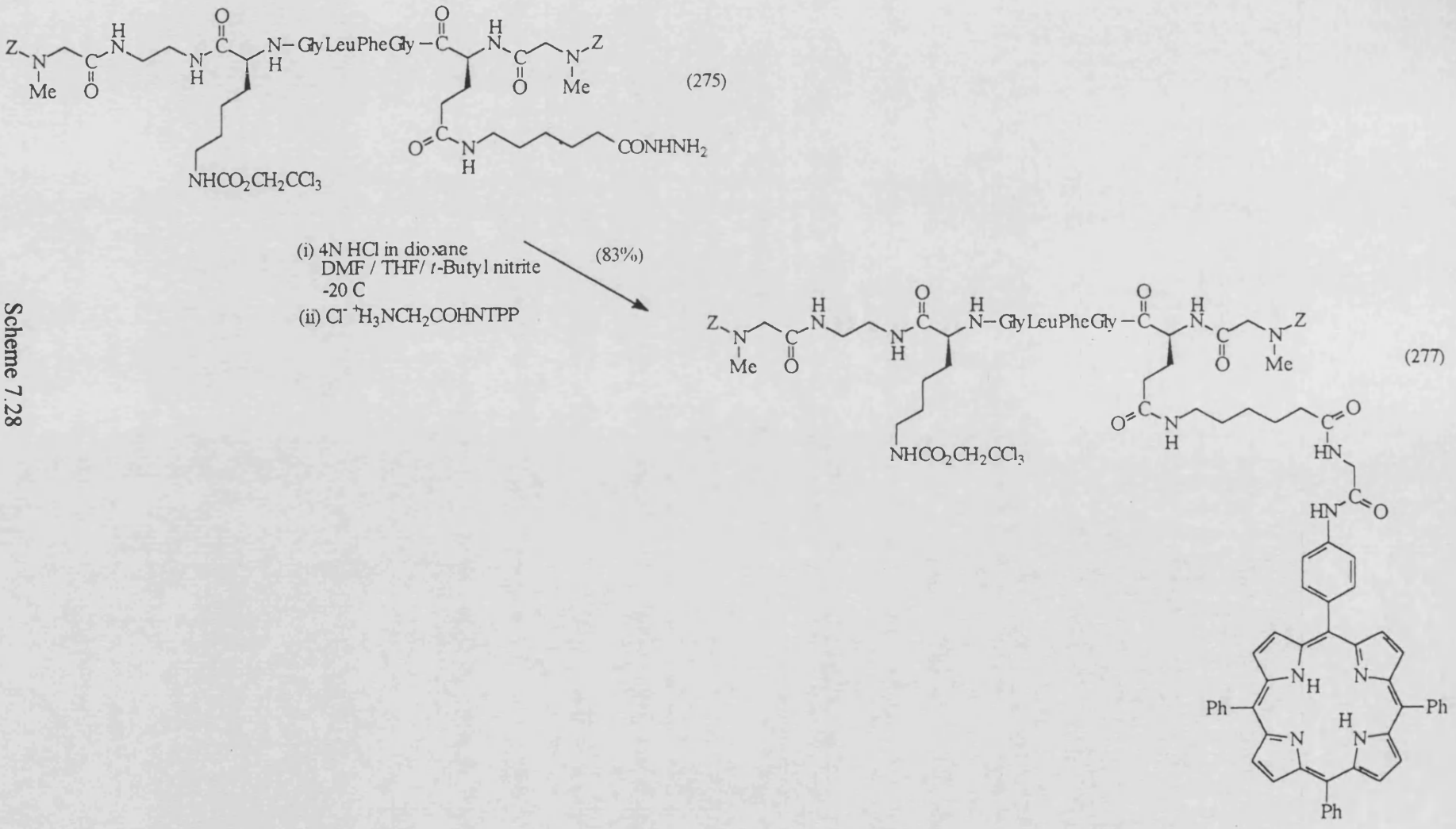
Scheme 7.26

Solubility problems were encountered with the large monomer containing both functionalised amino acids. Hydrazide formation was achieved on a suspension of the peptide in a large excess of hydrazine hydrate and did not affect the Troc protection of the lysine residue. Formation of the azide was slow, increased dilution of the reaction was required to enable solution. however, the azide was successfully formed and **(277)** prepared in 83% yield (Scheme 7.27 and Scheme 7.28).




Scheme 7.27

Thus, it is possible to couple a monofunctionalised TPP derivative to the prepared peptide monomers. The isocyanate synthon, *p*-nitrophenyl carbamate, is an effective electrophile for coupling to amine containing peptides or more universally to amine-containing drugs. Likewise, extension of the porphyrin, to give an aliphatic amine derivative, provides an effective nucleophile. Although in this work coupling to this amine is achieved through an azide method, coupling would be possible using any peptide coupling method.


(275)
(i) 4N HCl in dioxane
DMF / THF/ t-Butyl nitrite
-20 C
(ii) Cl⁻ ⁺H3NCH2COHNTPP
(83%)
(277)

Scheme 7.28

# CHAPTER EIGHT

# MODEL REACTIONS

## 8.1 Introduction

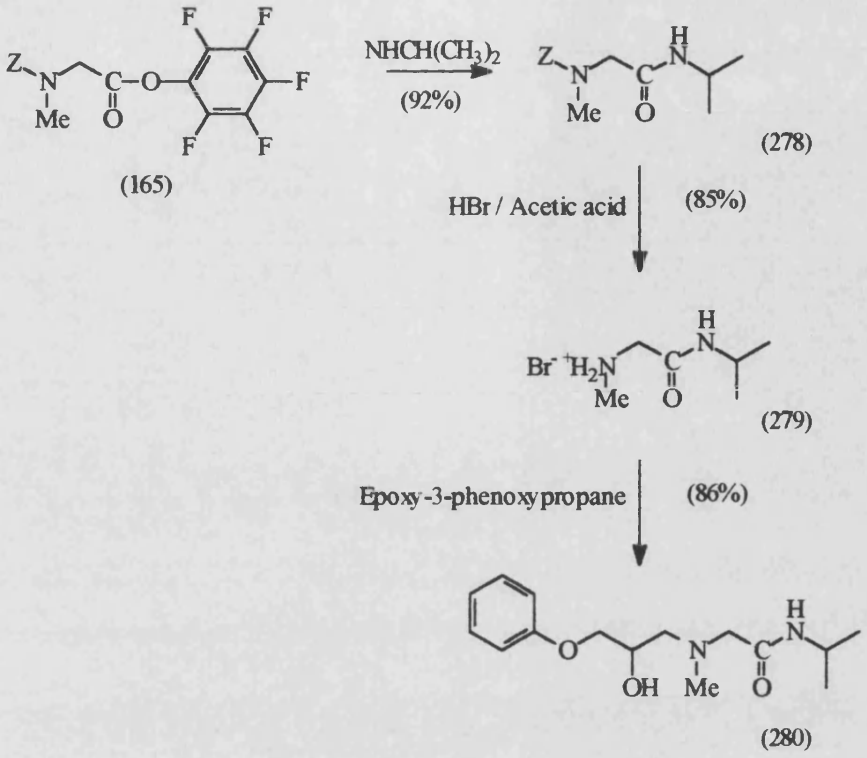
As discussed in earlier chapters, a number of monomers have been prepared, both degradable and non degradable, and either containing or not containing porphyrin. To prepare polymers from these monomers it is essential to remove the Z protecting group. The deprotected monomer can then be allowed to react with activated PEG to form a polymer. Following polymerisation, incorporation of manganese, in order to measure the relaxivity of the polymer-porphyrin conjugates, must be achieved.

In this chapter, a number of model reactions are reported. Successful deprotection of both types of monomers has been achieved. PEG activation, as the diglycidyl ether, has also been accomplished. Model polymerisation results have demonstrated the feasibility of the intended polymerisation reaction between a bis (secondary amine) and a bis diglycidyl ether derivative of PEG.

## 8.2 Polymerisation Investigations

### 8.2.1 Reaction between sarcosine and an epoxide

In the first instance, it was essential to confirm that the secondary amine of sarcosine would react with an epoxide. ZSarOPFP was allowed to react with a simple amine, isopropylamine (Scheme 8.1). This compound was then deprotected and treated with the model epoxide, epoxy-3-phenoxypropane.




Scheme 8.1

Deprotection of Z protected peptides can be achieved by numerous methods. The most well known method is catalytic hydrogenolysis with palladium on charcoal. Many solvents can be used to perform the reaction. Methanol and ethanol are the most easy to use, however, solvents such as acetic acid, acetic acid and water mixtures and DMF can be used where the peptide is of low solubility [606].

Another popular approach is treatment of the peptide with hydrogen bromide in acetic acid (HBr / Acetic acid) [274,287]. This was developed by Ben Ishai following from reports that hydrogen iodide was successful in the removal of Z groups. It is particularly attractive, as it can be used to remove Z groups selectively in the presence of benzyl esters and can afford the deprotection of sulphur-containing peptides which are known to poison hydrogenation catalysts. Unfortunately, the hydrobromide salts prepared by this route are highly hygroscopic. Alternatively, Lewis acids, such as trimethylsilyl iodide and boron tribromide, can be used to afford the cleavage of Z groups [265].

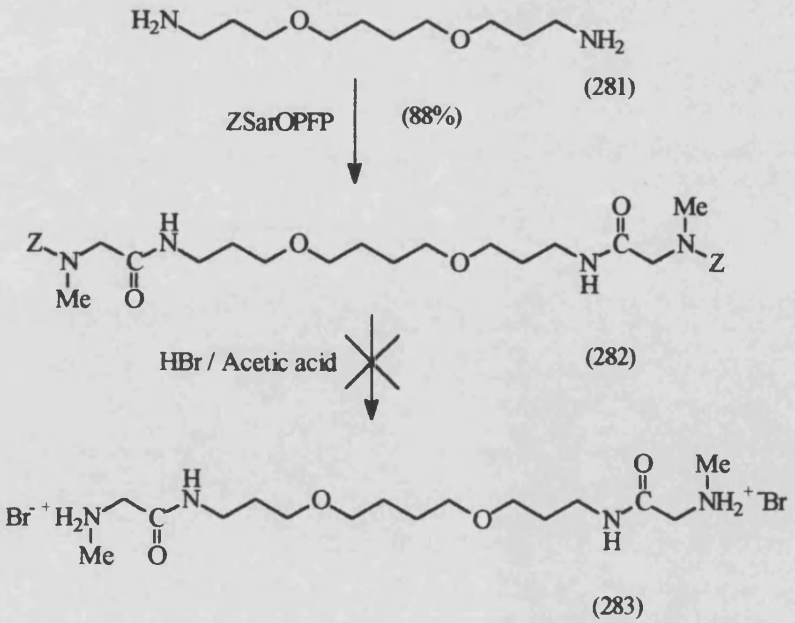
Sodium in liquid ammonia [267,288] can also be used for the removal of Z groups. However, this harsh method is usually confined to situations where both a Z and Tosyl group are to be removed from a peptide.

In this model reaction, deprotection was achieved using the HBr / Acetic acid method. to give the hydrobromide salt in 85% yield. Treatment of this amine with epoxy-3-phenoxypropane in ethanol and base gave **(280)** in 86% yield. This confirmed the feasibility of the reaction on which polymerisation was to be based (Scheme 8.1) .

8.2.2 Model Polymerisation

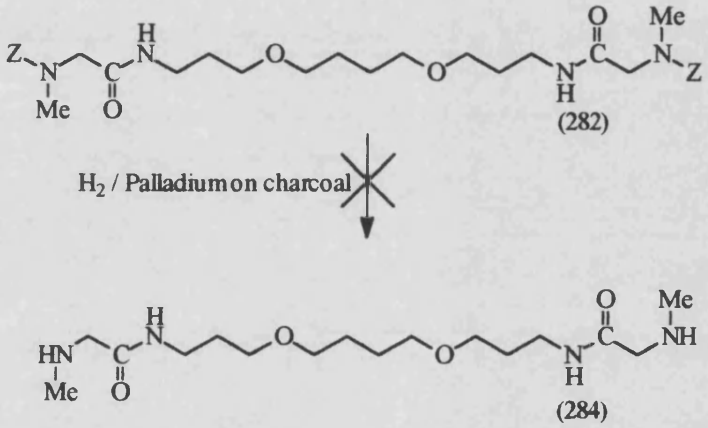
8.2.2.1 Model Bis-Amine Monomers

In order to extend these studies further, a model bis-amine was prepared to resemble the peptide monomers. Initially the model compound chosen was 4,9-dioxa-1,2-dodecane-1,12-diamine. This was successfully treated with ZSarOPFP to give the α, ω bis-protected model compound in 88% yield (Scheme 8.2).



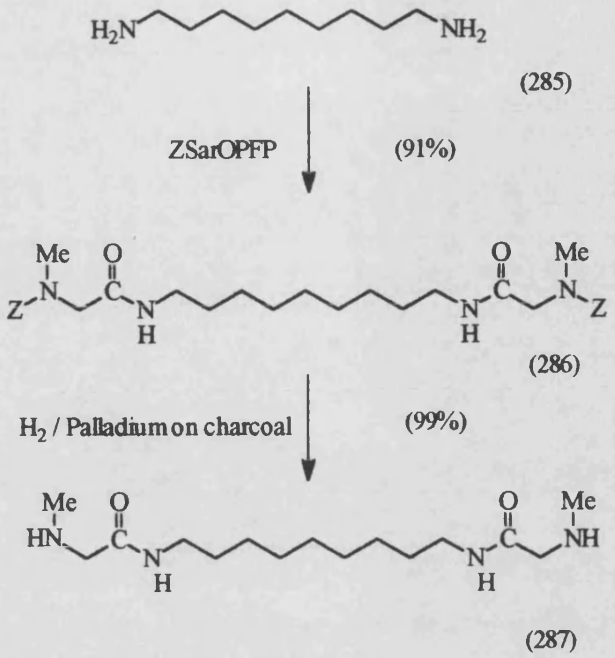

Scheme 8.2

Deprotection of this compound proved troublesome. Treatment with HBr / Acetic acid resulted in the formation of two products, one of which was the desired deprotected compound and one that was unidentifiable. As prolonged treatment had been required to afford transformation of the starting material, cleavage of the ether bonds may have occurred. In a second approach, the model compound was subjected to catalytic hydrogenolysis. Treatment in THF with hydrogen and palladium gave a number of products that were not identifiable. To investigate the reaction further, the products formed on hydrogenolysis were reprotected with Z. In this reprotection, a number of products were again formed although, none of the original starting material was formed. This suggested that catalytic hydrogenolysis also resulted in the cleavage of the ether bonds.




Scheme 8.3

Thus a second model compound was chosen; nonane-1,9-diamine. This was easily transformed to a diprotected α,ω-secondary amine on treatment with ZSarOPFP (91%). This was then treated, in methanol, with hydrogen and catalyst to give the unprotected α,ω-methylamino model in 99% yield (Scheme 8.4).



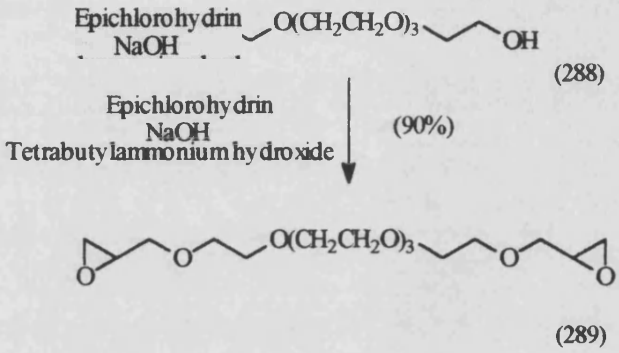

Scheme 8.4

8.2.2.2. Activation of PEG

A α,ω-bis-electrophilic PEG derivative was then required to facilitate a model polymerisation study. The formation of diglycidyl ethers from diols and epichlorohydrin is well known but sometimes difficult to achieve [290]. By-products can be formed, either the monoglycidyl ethers or oligomers. Simple methods [289] using epichlorohydrin and base require prolonged treatment at elevated temperatures e.g. 11 hours at 60°C. However, Gu *et al* [290] developed a method in which phase transfer catalysts, such as crown ethers and quaternary ammonium salts, could be used to enable fast and efficient transformation of diols to diglycidyl ethers using epichlorhydrin and sodium hydroxide. Quaternary ammonium salts, such as tetrabutylammonium hydrogen sulphate, were most efficient.

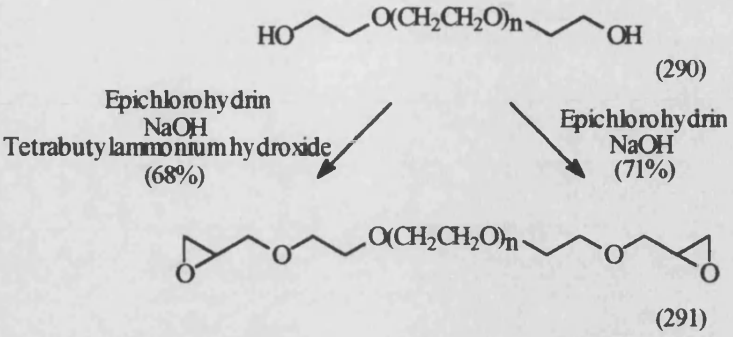
Following a modification of this method, the α,ω-bis-(glycidyl ether) of pentaethylene glycol was prepared in 90% yield (Scheme 8.5). However, using the published work-up procedures of this method, the phase transfer catalyst is likely to remain, in part, in the product. This was unacceptable, as it could interfere in the polymerisation reaction

and would be difficult to remove from any resulting polymer. Thus an alternative method was required. Gu *et al.*, had demonstrated that, better results were obtained with higher chain-length glycols, in comparison to short chains, even in the absence of phase transfer catalyst. These results were further improved by addition of the catalyst. This was proposed to be due to increased solubilisation of the catalyst in epichlorohydrin as the molecular weight of the diol increases. However, other workers [291,292], have proposed that PEG can itself act as a phase transfer catalyst. This is not surprising as crown ethers, which also contain repeating $OCH_2CH_2$ units, are effective catalysts. Thus, these workers were able to prepare a number of α,ω-diglycidyl ethers using PEG 400 as a catalyst.




Scheme 8.5

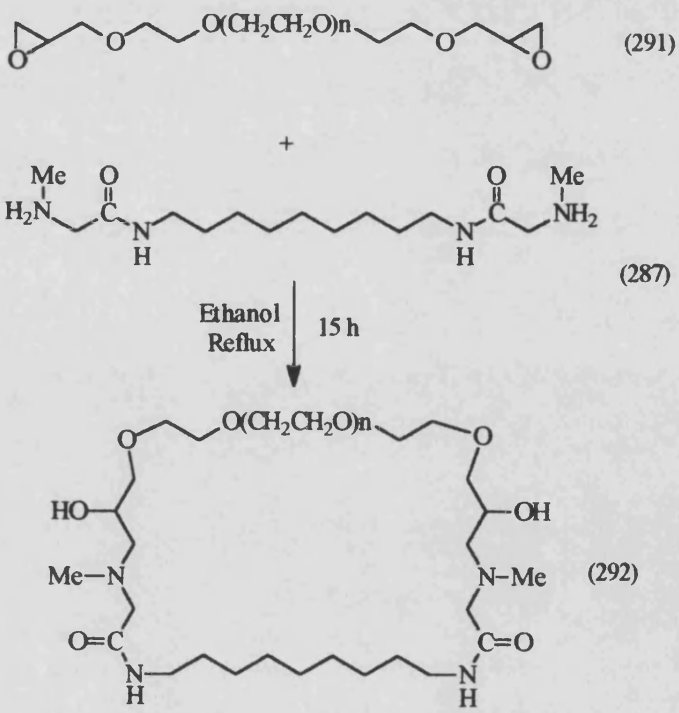
Therefore, it is possible that the effects seen by Gu *et al.* [290], with the long chain glycols, are due to self-catalysis. To investigate this possibility, the diglycidyl ether of PEG 400 was prepared using two methods (Scheme 8.6). In the first case, the derivative was prepared using the catalyst tetrabutylammonium hydroxide and, in the second case, using the same conditions but without the catalyst. Using the catalyst, the diglycidyl ether was successfully prepared. The derivative was also prepared without inclusion of the catalyst, although recovery from the reaction mixture was reduced, giving a cleaner product. This confirmed the ability of PEG 400 to act as a catalyst and thus a simpler and cleaner method for the preparation of α,ω-diglycidyl ethers of PEG is available.




Scheme 8.6

8.2.2.3 Polymerisation

A model polymerisation reaction was then performed, between the activated PEG 400, prepared without catalyst, and the model bis-amine **(287)** (Scheme 8.7). The two components were heated at reflux for 15 hours. NMR analysis of the reaction demonstrated the reaction to have reached completion. This was most elegantly seen in the transformation of the singlet signal of sarcosine $CH_2$ in the monomer to a pair of doublets, owing to the asymmetric environment, in the 'polymer'.




Scheme 8.7

To ascertain the extent of polymerisation and the role of ethanol-based chain termination, a sample was subjected to size-analysis using a size-exclusion gel permeation column. This analysis was performed by Dr Gareth Price, School of Chemistry, University of Bath. The results provided were compared with those for both the monomers. These results apparently conflict with the NMR interpretation of efficient polymerisation. Only small oligomers appeared to have been prepared. This is surprising, since if only small oligomers had been formed, a large number of 'end groups' (oxiranes and chiral $CH_2NHCH_3$ units) would remain which would be seen on the NMR spectrum.

However, electrospray mass spectral analysis reconciled the apparent dichotomy. A series of mass ions were seen which had an identical distribution to those seen with PEG 400. PEG 400 is composed of a number of PEG polymers with varying chain length; thus, with mass spectral analysis, a number of mass ions are seen which are related to the number of $CH_2CH_2O$ units in each fraction. However, with the sample from the 'polymerisation', these signals were of mass numbers corresponding to a macrocycle formed by the reaction of both epoxides of one PEG chain with both amines of one model monomer. This was unexpected as macrocycles are usually very difficult to synthesise, requiring high dilution conditions. However, in this reaction, the reagents were present in ~10% w/v concentration. The formation of the macrocycle is probably due to a property of the model bis-secondary amine, perhaps its lack of rigidity, or due to the relative length of the two components in solution, rather than an intrinsic problem with the polymerisation reaction. This could be further investigated by co-polymerising this bis-amine with PEG $\alpha,\omega$–diglycidyl ethers of different molecular weights.

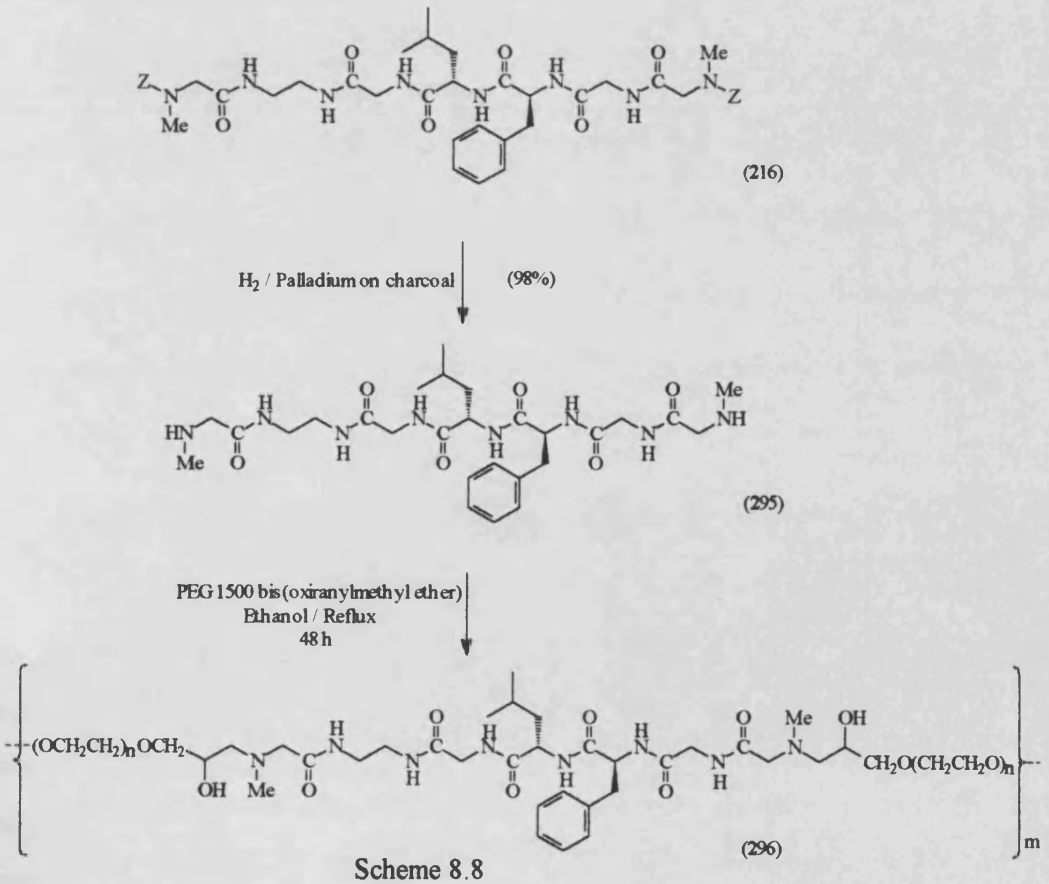
## 8.3 Polymerisation of Peptide Monomers

For the polymerisation of the peptide monomers, PEG of mean molecular weight 1500 was chosen as the co-monomer. This was selected as it is of a similar molecular weight to the peptide monomers. Thus, in the polymerisation, an approximately 1:1

molecular weight polymer will be formed. PEG 1500 was easily activated as the α,ω-diglycidyl ether by the method previously described.

In order to investigate the polymerisation fully, two peptide monomers were chosen to be polymerised. Firstly, the degradable but non-functionalised monomer **(216)** and secondly, the non-degradable lysine-porphyrin monomer **(252)**. In this way, the effects of all potentially problematic amino acid residues could be investigated.

Monomer **(216)** was deprotected using catalytic hydrogenolysis. As this compound was only sparing soluble in methanol, the reaction was heated to 60°C, prior to addition of the catalyst, to ensure dissolution. Complete loss of the protecting groups was observed after 30 hours. The deprotected compound was then allowed to react with the α,ω-diglycidyl ether derivative of PEG 1500 **(294)** in ethanol. After 48 hours, the reaction was halted by evaporation of the solvent (Scheme 8.8).




Scheme 8.8

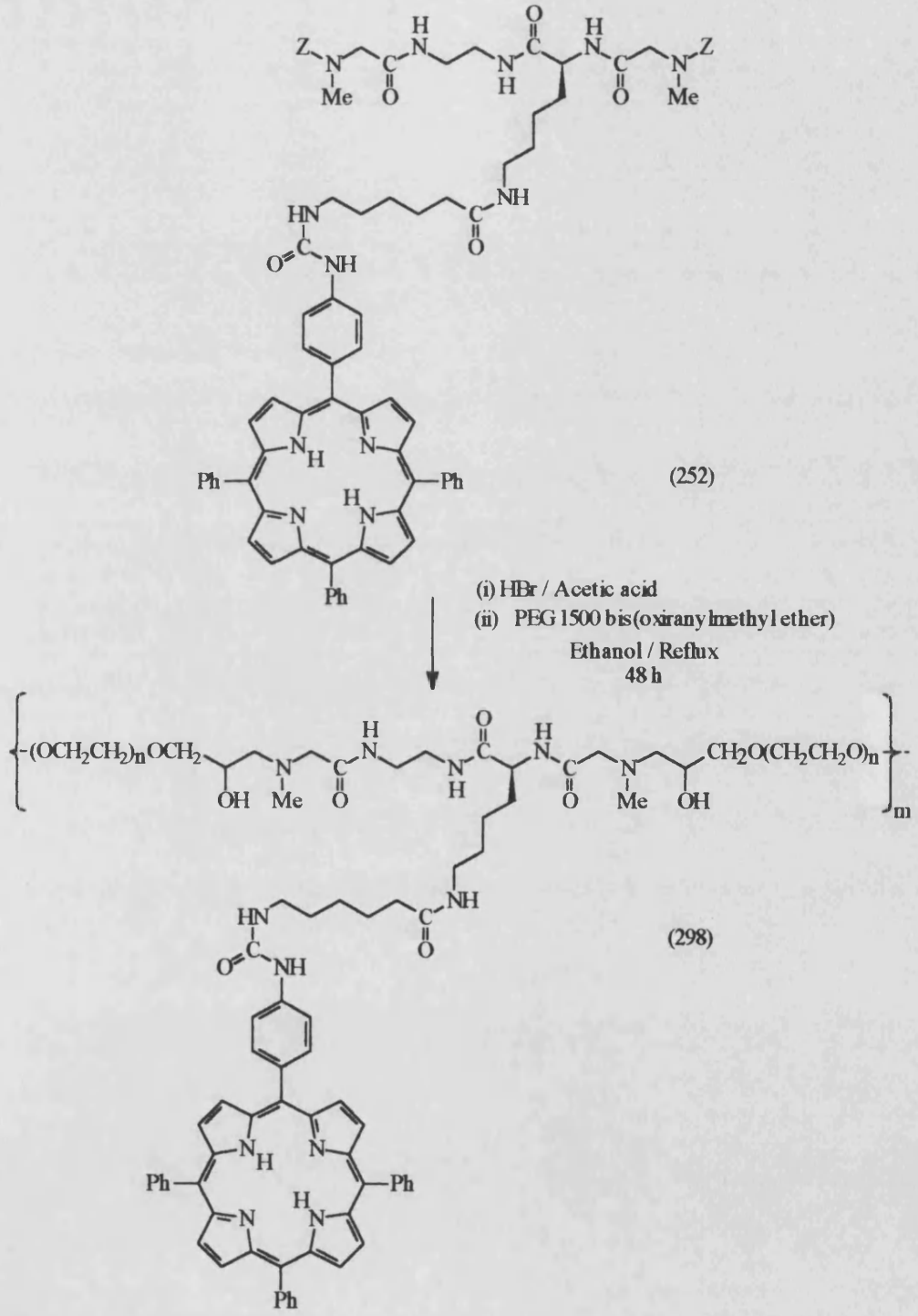
This product was subjected to electrospray mass spectral analysis. However, no signals were seen which related to the mass numbers of either the monomers or polymers. Equally, NMR analysis did not enable interpretation of polymerisation. Size exclusion analysis did however provide promising data. In comparison with a sample of the PEG 1500 α,ω-diglycidyl ether, a higher molecular weight population was seen with the purported polymer. This suggests that some polymerisation has occurred. Unfortunately, it appears that some of the epoxide remained unreacted. The degree of polymerisation is difficult to ascertain, although it is likely that only small molecular weight polymers have been formed.

With the porphyrin monomer, deprotection was more difficult. The compound was of low solubility in many simple solvents suitable for hydrogenolysis, however, it was soluble in acetic acid. Treatment with hydrogen, in the presence of palladised charcoal, resulted in the slow conversion of starting material over 10 days. A number of unidentifiable products were prepared. Deprotection with HBr / Acetic acid was then investigated. This was highly successful, complete conversion being observed after 45 minutes; the product precipitated on addition of ether (Scheme 8.9). NMR analysis showed the porphyrin to be protonated at the pyrrolic nitrogens suggesting that the product was present as the tetrahydrobromide salt.

The deprotected compound was then allowed to react with PEG 1500 diglycidyl ether in boiling ethanol. As the monomer was present as its hydrobromide, five equivalents of inorganic base ($K_2CO_3$) were also added to the polymerisation. After 48 hours, the solution was filtered to remove the base and the solvent was evaporated to halt the reaction.

As with the degradable monomer, electrospray mass spectral analysis and NMR analysis were unsuccessful in determining the extent of polymerisation. Analysis using size exclusion chromatography was also unable to provide confirmation of polymerisation. For the purported polymer, the majority of the population was of similar size to the monomeric PEG 1500 α,ω-diglycidyl ether. Although this may

mean that polymerisation was unsuccesful, it is more likely due to an artifact of the porphyrin in the GPC system. The solubility of the polymer in the eluting solvent, THF, would be expected to be enhanced in the presence of a porphyrin.



## 8.4 Incorporation of Manganese

In a final step, the incorporation of manganese into the porphyrin was investigated. Although, for the polymers, metallation will be the last step following polymerisation, the method was developed using deprotected monomers.

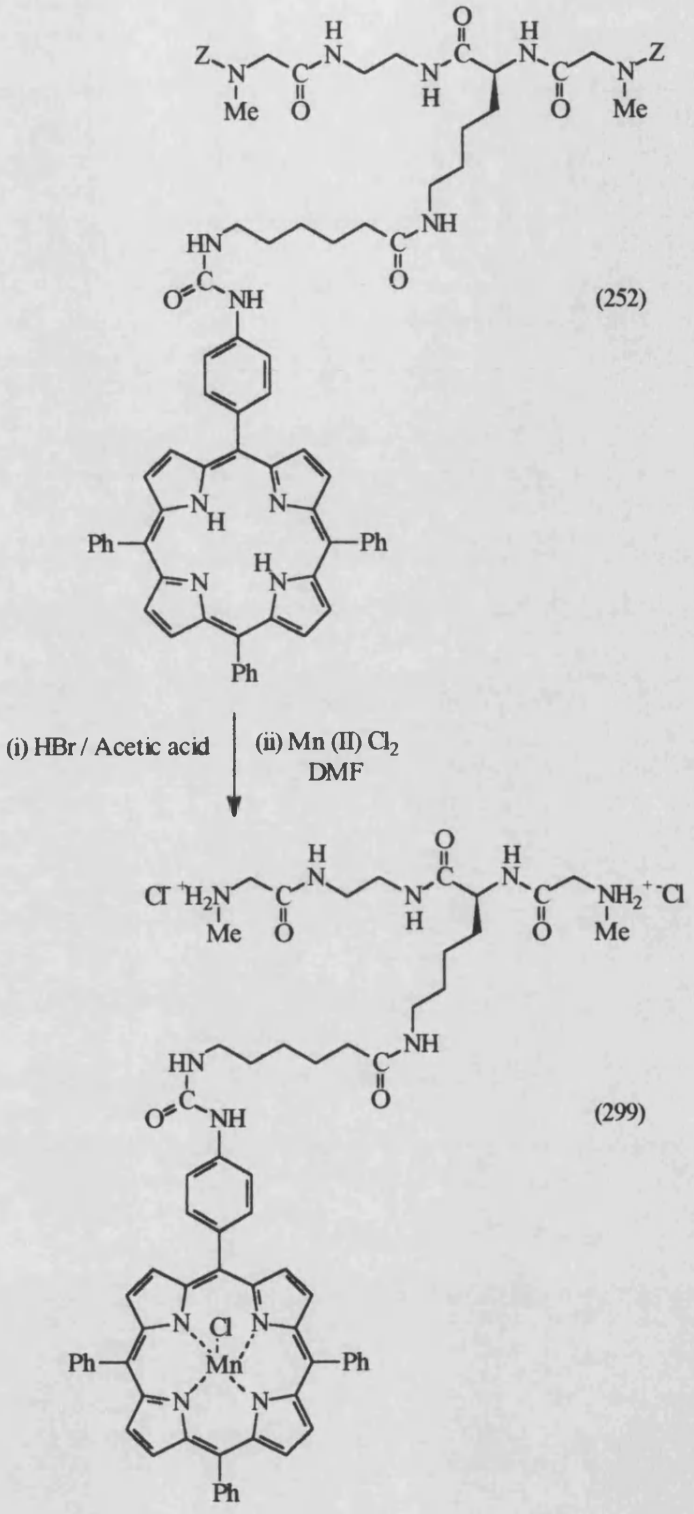
The metallation of porphyrins can be achieved in many ways, dependant on the ion to be chelated. Lawley and Threadgill [293] investigated the preparation of a manganese derivative of TPP. In a modification of the method of Kennedy and Murray, TPP was heated at reflux with manganese (II) acetate in DMF. The resulting chelate was treated with brine to replace the labile acetate ligand with the more stable chloride ligand. However, they were unable to isolate the product without concomitant precipitation of sodium chloride.

In a different approach following the method of Adler *et al.* [294], the nondegradable lysine-porphyrin monomer was treated with manganese (II) chloride in DMF (Scheme 8.10). At reflux both the monomer and the metal are sufficiently soluble to allow chelation to take place. The reaction was followed by the UV spectrum of the reaction mixture. The starting material had a UV spectrum similar to that of TPP, *i.e.* an etio type spectrum. Thus, a strong Soret band can be seen and in the visible region four smaller bands decreasing in intensity with increasing wavelength. As metallation occurred a shift in the Soret band was observed along with the disappearance of signal I and a change in the structure of peaks II, III and IV. Metallation was extremely slow, therefore numerous equivalents of manganese (II) chloride were added. After 30 hours the reaction was considered to have reached completion. As this compound cannot be analysed by NMR, owing to the presence of the paramagnetism, mass spectral analysis was undertaken. Unfortunately none of the mass ions obtained were identifiable either as expected product or fragments. Therefore, although metallation has occurred, as shown by UV, no information has been obtained on the integrity of the peptide. As this method involved prolonged heating in DMF, it is possible that the peptide has been damaged.

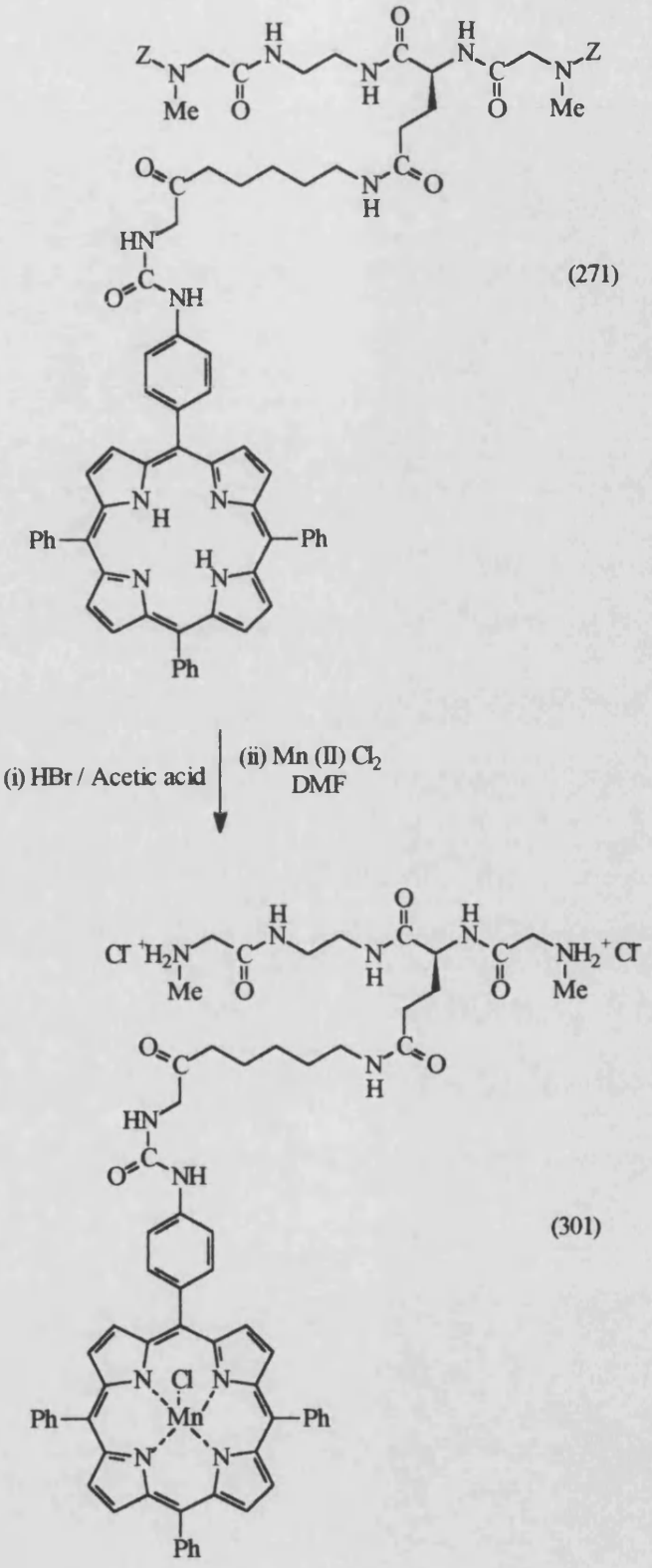
In a second approach, the same method was used, except that a large excess of manganese (II) chloride was added initially and the temperature was carefully maintained to ensure reflux (Scheme 8.11). In this case, the non-degradable glutamic acid-porphyrin monomer was investigated. After 2 hours, similar changes in the UV spectrum were observed and the reaction was halted. Again no information could be obtained by mass spectral analysis.

Thus it is essential for further work to be performed on this metallation reaction, firstly, to ensure the peptide remains intact and, secondly, to confirm that no free manganese (II), which could alter the apparent relaxivity of the polymer preparation, is present.

From these model reactions, it is apparent that both polymerisation and metallation of the monomers will be easily achieved.




Scheme 8.10




Scheme 8.11

# CHAPTER NINE

## CONCLUSION

Synthetic strategies have been successfully developed for the preparation of a co-polymer of poly(ethylene glycol) and a biodegradable peptide unit and a conjugate of this polymer with the potential contrast enhancement agent chlorotetraphenyl-porphyrinatomanganese (III)

Investigations towards the synthesis of a monofunctionalised derivative of tetraphenyl porphyrin demonstrated that the monoamine derivative can be easily prepared by the method of Kruper *et al* [257]. Initial attempts to extend or modify the functionalisation, by incorporation of an aliphatic spacer, resulted in compounds of limited solubility which were subsequently difficult to couple to the peptide monomers. However, activated monofunctionalised porphyrins were prepared, which could be coupled to the peptide monomers extended by a six-carbon chain spacer. Thus, for attachment of the porphyrin to a lysine residue, the electrophilic *p*-nitrophenyl carbamate was successfully prepared. To enhance the nucleophilicity of the amine derivative prior to coupling with an activate glutamic acid derivative, a glycine derivative has been prepared which provides a more nucleophilic aliphatic amine.

A number of peptide units have been prepared using suitable orthogonal protecting groups and an active ester coupling strategy. Ethane-1,2-diamine has been used as a retro-inverso sequence. This allows the coupling of sarcosine at both termini of the peptide to provide the α,ω-bis(secondary amine) monomer required for polymerisation with the α,ω-bis(glycidyl ether) derivatives of PEG. Initially, three non-degradable monomers were prepared in which only one amino acid, glutamic acid or lysine, was present. these monomers have suitable functionality for the attachment of porphyrin. A further monomer has been prepared in which the degradable sequence is present but no functionalised amino acid. These monomers are useful as models for

coupling and polymerisation reactions and for comparative purposes in degradability studies.

Four peptide monomers have been prepared in which the degradable unit and a functionalised amino acid are present. In two sequences, however, lysine is present; in one it is situated at the N-terminus of the GlyPheLeuGly sequence and in the other at the C-terminus. The lysine moiety in these monomers has been deprotected and extended *via* a spacer unit. In a third monomer glutamic acid has been incorporated at the N-terminus. The glutamic acid is derivatised with the spacer, prior to incorporation into the peptide sequence. In an alternative approach, a degradable monomer has been prepared in which both derivatised glutamic acid and lysine are incorporated. This offers great potential, both for increasing the loading of the porphyrin onto the peptide and for the incorporation of a targeting moiety to the polymer. this monomer has also been successfully deprotected at the lysine ε-amine to prepare a monomer bearing two six-carbon spacer units.

Nine porphyrin-peptide conjugates have been prepared. With monomers containing lysine residues, treatment of either the deprotected lysine or the spacer-extended lysine with the electrophilic porphyrin derivative yielded conjugates linked *via* a urea bond. With glutamic acid containing monomers, the peptide was easily activated as the azide to couple efficiently to the enhanced nucleophilic porphyrin derivative. With the monomer incorporating both glu and lys residues, conjugates have been prepared in which the porphyrin is attached either to the lysine or glutamic acid.

Model polymerisation reactions have demonstrated the suitability of the proposed polymerisation reaction between a diglycidyl ether and a secondary amine. PEG of various chain lengths has been activated as the α,ω-bis(glycidyl ether). During model studies polymerisation of the activated PEG 400 with sarcosine N-(9-(sarcosyl-amino)nonyl)amide resulted in the formation of a macrocycle. However, in preliminary polymerisation studies with the GlyPheLeuGly monomer and PEG 1500, linear polymer is believed to have been formed. Initial experiments on the

incorporation of manganese into simple porphyin-peptide monomers have demonstrated chelation of the metal. Further evaluation of both polymerisation and metallation is, however, required.

# EXPERIMENTAL

## General Procedure

IR spectra were recorded as liquid films using a Perkin Elmer 782 instrument. UV spectra were obtained of solutions in methanol with a Perkin Elmer Lambda 3 spectrometer. $^{1}H$ and $^{19}F$ NMR data were recorded on a Jeol GX270 spectrometer (270.05 MHz for $^{1}H$) and a Jeol EX400 (399.65 MHz for $^{1}H$ and 376 MHz for $^{19}F$). Tetramethylsilane was used as an internal standard for samples dissolved in $CDCl_3$ and $(CD_3)_2SO$. $^{19}F$ chemical shifts are referenced to external fluorotrichloromethane. Multiplicities are indicated as follows s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet). Where two sets of data are listed, the compound exists as discrete rotamers at ambient temperature. COSY spectra were used to assign signals in the $^{1}H$ spectra where stated. Mass spectra were obtained by electron impact (EI), chemical ionisation (CI), fast atom bombardment (FAB) or electrospray using a VG7070 spectrometer and are reported in the form *m/z* for selected ions.

Thin layer chromatography was carried out using Merck Kieselgel 60F plates, visualisation was achieved by UV light and ninhydrin. Solvents systems were :

A : $CH_2Cl_2$ : Hexane (1 : 1)
B : $CH_2Cl_2$
C : $CHCl_3$ : MeOH (9 : 1)
D : $CH_2Cl_2$ : $Et_2O$ (20 : 1)
E : $Et_2O$ : MeOH (1 : 1)
F : $CHCl_3$ : MeOH (19 : 1)
G : $CHCl_3$

Flash chromatography was performed using Merck Silica Gel 60 (0.040-0.063 mm) flash silica. Melting points are uncorrected. All chiral amino acids are (L), unless otherwise stated.

Unless otherwise stated all solutions were dried with $MgSO_4$ and all solvents evaporated under reduced pressure. The following abbreviations have been used throughout the text: $CH_2Cl_2$ (dichloromethane), $CHCl_3$ (chloroform), $Et_2O$ (diethyl ether). EtOAc (ethyl

acetate), DCC (N,N'-dicyclohexylcarbodiimide), DMF (dimethylformamide), brine (saturated aqueous sodium chloride), DMAP (4-dimethylaminopyridine), MeOH (methanol), THF (tetrahydrofuran).

**5-(4-Nitrophenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (90)**

Fuming nitric acid (d. 1.5 g $ml^{-1}$) (2.26 ml) was added stepwise during 2 h to a solution of 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine (2.00 g, 3.26 mmol) in ethanol-free $CHCl_3$ (300 ml). After a further 1 h, the solution was washed five times with water (300 ml). The solution was dried with a mixture of $Na_2CO_3$ and $MgSO_4$ and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / hexane 3:2) gave 5-(4-nitrophenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (1.17 g, 55%) as a purple solid. Mp >300°C

$^1$H NMR ($CDCl_3$)

δ -2.75 (2 H, s, *21,23*-$H_2$), 7.75 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.20 (6 H, m, 3 × Ph 2,6-$H_2$), 8.36 (2 H, d, $J$ = 8.3 Hz, disubstituted aromatic-$H_2$), 8.60 (2 H, d, $J$ = 8.7 Hz, disubstituted aromatic-$H_2$), 8.71 (2 H, d, $J$ = 4.8 Hz, *2,3,7,8,12,13,17,18*-$H_2$), 8.87 (6 H, m, *2,3,7,8,12,13,17,18*-$H_6$).

T.L.C. (A) Rf = 0.38

**4-(10,15,20-Triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (93)**

Tin (II) chloride (0.595 g, 2.64 mmol) was added to 5-(4-nitrophenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (580 mg, 0.88 mmol) in aq. HCl (9M, 20 ml). The solution was heated at 65°C for 2 h and then allowed to cool. The solution was added to water (70 ml) and taken to pH 8 by the addition of concentrated aq. ammonia. The suspension was extracted nine times with $CHCl_3$. The organic layer was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / hexane 5:1) gave 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (462 mg, 84%) as a purple solid. Mp > 300°C

$^1$H NMR ($CDCl_3$)

δ -2.76 (2 H, s, *21,23*-$H_2$), 3.98 (2 H, br, $NH_2$), 7.05 (2 H, d, $J$ = 8.6 Hz, disubstituted aromatic-$H_2$), 7.77 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.02 (2 H, d, $J$ = 8.2 Hz, disubstituted

aromatic-$H_2$), 8.26 (6 H, m, 3 × Ph 2,6-$H_2$), 8.87 (6 H, m, *2,3,7,8,12,13,17,18*-$H_6$), 8.98 (2 H, d, $J$ = 4.9 Hz, *2,3,7,8,12,13,17,18*-$H_2$)

T.L.C. (B) Rf = 0.53

**4-Oxo-4-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)butanoic acid (105)**

Succinic anhydride (79 mg, 0.79 mmol) was added to 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (500 mg, 0.79 mmol) in ethanol-free $CHCl_3$ (20 ml). The mixture was boiled under reflux for 4 h. The mixture was cooled to ambient temperature and the precipitated solid was collected by filtration to give 4-oxo-4-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)butanoic acid (502 mg, 87%) as a purple solid. Mp > 300°C

$^1$H NMR ($CDCl_3$)

δ - 2.9 (2 H, s, *21,23*-$H_2$), 2.67 (2 H, d, $J$ = 5.5 Hz, NHCOC$\underline{H_2}$C$H_2$C$O_2$H), 2.74 (2 H, d, $J$ = 5.5 Hz, NHCOC$H_2$C$\underline{H_2}$C$O_2$H), 7.82 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.06 (2 H, d, $J$ = 8.8 Hz, disubstituted aromatic-$H_2$), 8.15 (2 H, d, $J$ = 8.8 Hz, disubstituted aromatic-$H_2$), 8.23 (6 H, m, 3 × Ph 2,6-$H_2$), 8.82 (6 H, m, *2,3,7,8,12,13,17,18*-$H_6$), 8.91 (2 H, d, $J$ = 4.7 Hz, *2,3,7,8,12,13,17,18*-$H_2$), 10.46 (1 H, s, NH), 12.06 (1 H, br, OH)

T.L.C. (C) Rf = 0.67

MS (FAB+) 730 (M + H).

Microanalysis $C_{48}H_{33}N_5$ Found C 77.1% H 4.69% N 9.3% Calc'd. C 78.99% H 4.83% N 9.59%.

**N-Butyl-N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)butanediamide (110)**

4-Oxo-4-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)butanoic acid (493 mg, 0.68 mmol) was treated with oxalyl chloride (345 mg, 2.72 mmol) in THF (20 ml) and DMF (1.00 ml) for 1 h. The solvent and excess reagent were evaporated and the residue in THF (20 ml) was treated with butylamine (303 mg, 4.15 mmol). The

solution was stirred for 24 hours and then the solvent and excess reagent was evaporated. Chromatography ($Et_2O$ : EtOAc 1:1) gave a material characterised as 5-(4-succinimido)phenyl-10,15,20-triphenyl-21*H*,23*H*-porphine as a purple glass (150 mg, 21%).

$^1$H NMR ($CDCl_3$)

δ -2.78 (2 H, s, *21,23*-$H_2$), 3.03 (4 H, s, NCOC$\underline{H_2}$C$\underline{H_2}$CO$_2$), 7.70 (2 H, d, $J$ = 8.5 Hz, disubstituted aromatic-$H_2$), 7.78 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.21 (6 H, m, 3 × Ph 2,6-$H_2$), 8.32 (2 H, d, $J$ = 8.0 Hz, disubstituted aromatic-$H_2$), 8.85 (8 H, m, *2,3,7,8,12,13,17,18*-$H_8$)

T.L.C. (C) Rf = 0.81

**Methyl 6-oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)-hexanoate (114)**

Hexanedioic acid monomethyl ester (1.00 g, 6.24 mmol) in $CH_2Cl_2$ / DMF was treated with oxalyl chloride (1.58 g, 12.49 mmol) and the solution was stirred for 18 h. The solvent and excess reagent were evaporated. A portion of the acid chloride (1 mmol) was added to 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (629 mg, 1.00 mmol) and N,N-diisopropylethylamine (258 mg, 1.00 mmol) in $CH_2Cl_2$ (20 ml). The solution was stirred for 24 h and was then washed with water, cold 10% aq. $H_2SO_4$ and 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated to give methyl 6-oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)hexanoate (620 mg, 80%) as a purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.79 (2 H, s, *21,23*-$H_2$), 1.86 (4 H, m, $CH_2C\underline{H_2}C\underline{H_2}CH_2$), 2.44 (2 H, t, $J$ = 6.8 Hz $COC\underline{H_2}CH_2CH_2C\underline{H_2}CO$), 2.53 (2 H, t, $J$ = 6.8 Hz, $COC\underline{H_2}CH_2CH_2C\underline{H_2}CO$), 3.82 (3 H, s, $OCH_3$) 7.75 (9 H, m, 3 × Ph 3,4,5-$H_3$), 7.91 (2 H, d, $J$ = 8.3 Hz, disubstituted aromatic-$H_2$), 8.14 (2 H, d, $J$ = 8.3 Hz, disubstituted aromatic-$H_2$), 8.22 (6 H, m, 3 × Ph 2,6-$H_2$), 8.84 (6 H, m, *2,3,7,8,12,13,17,18*-$H_6$), 8.87 (2 H, d, $J$ = 4.9 Hz, *2,3,7,8,12,13,17,18*-$H_2$)

T.L.C. (C) Rf = 0.83

MS (FAB+) 772 (M + H)

**6-Oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)hexanoic acid (115)**

Methyl 6-oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)hexanoate (530 mg, 0.69 mmol) was stirred at reflux with NaOH (500 mg, 12.5 mmol) in MeOH (40 ml) and water (2 ml) for 5 d. Hydrochloric acid (9 M, 1.25 ml) was added and the suspension was filtered to remove precipitated NaCl. The solvent was evaporated from the filtrate to give a purple glass which was shown by MS to comprise a mixture of the educt and the product.

MS (FAB+) 772 (M+H for ester), 758 (M+H for acid).

**Methyl 6-aminohexanoate hydrochloride (117)**

Thionyl chloride (10 ml) was added slowly to a suspension of 6-aminohexanoic acid (6 g, 45.8 mmol) in MeOH (50 ml) and the resulting solution was stirred for 2 d. The solvent and excess reagent were evaporated to give methyl 6-aminohexanoate hydrochloride (8.32 g, quantitative) as a white crystalline solid. Mp 81-83°C

$^1$H NMR ($CDCl_3$)

δ 1.45 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.64 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.73 (2 H, br, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.34 (2 H, t, $J$ = 7.2 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.03 (2 H, br, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.67 (3 H, s, $CH_3$), 8.24 (3 H, br, $NH_3^+$)

T.L.C. (C) Rf = 0.12

**Methyl 6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)-hexanoate (118)**

Phosgene in toluene (20%, 2.22 ml, 4.2 mmol) was added to 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (1.32 g, 2.1 mmol), triethylamine (850 mg, 8.4 mmol) in $CH_2Cl_2$. The solution was stirred for 6 h. The solvent and excess reagent were evaporated. The residue was dissolved in $CH_2Cl_2$. Methyl 6-aminohexanoate hydrochloride (381 mg, 2.10 mmol) and triethylamine (213 mg, 2.10 mmol) were added. The solution was stirred for 24 h and the solvent was evaporated. Chromatography ($CH_2Cl_2$ then $CH_2Cl_2$ / MeOH 40:1) gave methyl 6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoate (1.04 g, 62%) and 6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoic acid (286 mg, 17%) as purple glasses.

$^1$H NMR ($(CD_3)_2SO$)

δ -2.90 (2 H, s, *21,23*-$H_2$), 1.38 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.57 (4 H, m, $NCH_2C\underline{H_2}CH_2C\underline{H_2}CH_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.19 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.35 (3 H, s, $OCH_3$), 6.37 (1 H, m, $N\underline{H}CH_2$), 7.83 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic-$H_2$), 8.07 (2 H, d, $J$ = 8.4 Hz, disubstituted aromatic-$H_2$), 8.22 (6 H, m, 3 × Ph 2,6-$H_2$), 8.82 (6 H, br, *2,3,7,8,12,13,17,18*-$H_6$) 8.92 (2 H, t, $J$ = 4.7 Hz, *2,3,7,8,12,13,17,18*-$H_2$), 12.06 (1 H, s, NHCO)

T.L.C. (C) Rf = 0.65

$^1$H NMR ($CDCl_3$)

δ -2.90 (2 H, s, *21,23*-$H_2$), 1.26 (2 H, m, $NCH_2C\underline{H_2}C\underline{H_2}CH_2CH_2$), 1.55 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.23 (2 H, t, $J$ = 7.9 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.19 (2 H, q, $J$ = 7.9 Hz, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 6.45 (1 H, m, $N\underline{H}CH_2$), 7.32 (2 H, d, $J$ = 7.9 Hz, disubstituted aromatic-$H_2$), 7.59 (9 H, m, 3 × Ph 3,4,5-$H_3$), 7.88 (2 H, d, $J$ = 8.0 Hz, disubstituted aromatic-$H_2$), 8.12 (6 H, m, 3 × Ph 2,6-$H_2$), 8.82 (8 H, m, *2,3,7,8,12,13,17,18*-$H_8$)

T.L.C. (C) Rf = 0.27

**N-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)leucine methyl ester hydrochloride (122)**

2,4,5-Trichlorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (3.50 g, 8.52 mmol) was added to leucine methyl ester hydrochloride (1.55 g, 8.52 mmol), N,N-diisopropylethylamine (2.20 g, 17.04 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (30 ml). The solution was stirred for 4 d and 2,4,5-trichlorophenyl 6-(1,1-dimethylethoxycarbonyl-amino)hexanoate (1.75 g, 4.26 mmol) was added. The solution was stirred for 3 d and was then washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography (EtOAc / hexane 1:1) yielded N-(6-(1,1 dimethylethoxycarbonylamino)hexanoyl)leucine methyl ester (2.07 g, 68%) as a pale yellow oil.

$^1$H NMR ($CDCl_3$)

δ 0.87 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 0.91 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 1.35 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.42 (12 H, m, Bu-t, + Leu β-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.65 (4 H, m, Leu γ-H + Leu β-$H_2$, + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.22 (2 H, t, $J$ = 7.4 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.13 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.73 (3 H, s, $OCH_3$), 4.64 (2 H, m, Leu α-H + NH), 5.88 (1 H, d, $J$ = 7.9 Hz, Leu-NH)

T.L.C. (C) Rf = 0.34

**N-(6-Aminohexanoyl)leucine methyl ester hydrochloride (123)**

Hydrogen chloride was passed through N-(6-(1,1-dimethylethoxycarbonylamino)-hexanoyl)leucine methyl ester (1.91 g, 5.33 mmol) in $CH_2Cl_2$ for 20 min. The solvent and excess reagent were evaporated to give N-(6-aminohexanoyl)leucine methyl ester hydrochloride (1.57 g, quantitative) as a yellow oil.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.82 (2 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (2 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.29 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.55 (7 H, m, Leu β-$H_2$ + Leu γ-H + $NCH_2C\underline{H_2}CH_2C\underline{H_2}CH_2$),

2.11 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.69 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 4.25 (1 H, m, Leu α-H), 8.07 (3 H, br, $NH_3^+$), 8.25 (1 H, d, $J$ = 7.5 Hz, $N\underline{H}CH_2$)

T.L.C. (C) Rf = 0.12

**N-(6-(N'-(4-(10,15,20-Triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)-leucine methyl ester (124)**

Phosgene in toluene (20%, 0.72 ml, 1.36 mmol) was added to triethylamine (275 mg, 2.7 mmol) in $CH_2Cl_2$ (20 ml). 4-(10,15,20-Triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (426 mg, 0.68 mmol) was added during 10 min and the solution was stirred for 3 h. Phosgene in toluene (20%, 0.36 ml, 0.68 mmol) was added and stirring was continued for 1 h. Phosgene in toluene (20%, 1.08 ml, 2.04 mmol) was added and stirring continued for 1.5 h. The solvent and excess reagent were evaporated. The residue was dissolved in $CH_2Cl_2$ (20 ml). N-(6-Aminohexanoyl)leucine methyl ester hydrochloride (100 mg, 0.34 mmol) and triethylamine (69 mg, 0.68 mmol) were added. The solution was stirred for 24 h. Chromatography ($CH_2Cl_2$ / MeOH 15:1) gave N-(N-(N-(N-4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)ureido)-6-hexanoyl)leucine methyl ester (115 mg, 37%) as a purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.78 (2 H, br, *21,23*-$H_2$), 0.93 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.94 (3 H, d, $J$ = 5.9 Hz, Leu-$H_3$), 1.4-1.8 (6 H, m, $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.31 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.31 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.75 (3 H, s, $OCH_3$), 4.65 (1 H, m, Leu α-H), 6.03 (1 H, d, $J$ = 7.3 Hz, Leu NH), 6.96 (1 H, br, NH), 7.55 (2 H, d, $J$ = 8.1 Hz, disubstituted aromatic-$H_2$), 7.7 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.03 (2 H, d, $J$ = 8.4 Hz, disubstituted aromatic-$H_2$), 8.2 (6 H, m, 3 × Ph 2,6-$H_2$), 8.77 (2 H, d, $J$ = 4.8 Hz, *2,18*-$H_2$), 8.85 (4 H, m, *7,8,12,13*-$H_4$), 8.90 (2 H, d, $J$ = 4.8 Hz, *3,17*-$H_2$)

T.L.C. (C) Rf = 0.50

Acc. Mass 914.4409 (M + H) ($C_{58}H_{56}N_7O_4$ requires 914.4394)

**5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (125)**

4-Nitrophenyl chloroformate (1.36 g, 6.74 mmol) was added to 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (4.15 g, 6.74 mmol) and N,N-diisopropylethylamine (867 mg, 6.74 mmol) in $CHCl_3$ (50 ml). The solution was stirred for 24 h. The solvent was evaporated. Chromatography (EtOAc / hexane 1:3) yielded 5-(4-(4-nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (4.51 g, 86%) as a purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.73 (2 H, br, *21,23*-$H_2$), 7.36 (2 H, d, $J$ = 9.2 Hz, Ar-2,6), 7.76 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic-$H_2$), 8.22 (10 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic-$H_2$ + Ar-3,5), 8.84 (8 H, m, *2,3,7,8,12,13,17,18*-$H_8$)

T.L.C. (C) Rf = 0.95

**N-(1,1-Dimethylethoxycarbonyl)glycine pentafluorophenyl ester (126)**

N-(1,1-Dimethylethoxycarbonyl)glycine (59.14 g, 340 mmol) in EtOAc (300 ml) was cooled to 0°C. DCC (70.1 g, 340 mmol) and pentafluorophenol (62.6 g, 340 mmol) were added and the suspension was stirred for 48 h at 0°C. The suspension was filtered and the solvent was evaporated. The residue was dissolved in EtOAc and was filtered to give N-(1,1-dimethylethoxycarbonyl)glycine pentafluorophenyl ester (115.3 g, 99%) as a white wax.

$^1$H NMR ($CDCl_3$)

δ 1.46 (9 H, s, Bu-t), 4.28 (2 H, d, $J$ = 6.1 Hz, Gly-$H_2$), 5.12 (1 H, br, NH)

$^{19}$ F NMR ($CDCl_3$)

δ -152.7 (2 F, d, $J$ = 19 Hz, 2,6-Ar), -157.75 (1 F, t, $J$ = 23 Hz, 4-Ar), -162.4 (2 F, t, $J$ = 23 Hz, 3,5-Ar)

δ -153.2 (2 F, d, $J$ = 19 Hz, 2,6-Ar), -157.65 (1 F, t, $J$ = 23 Hz, 4-Ar), -162.3 (2 F, t, $J$ = 23 Hz, 3,5-Ar)

T.L.C. (C) Rf = 0.95

**5-(4-(N-(1,1-Dimethylethoxycarbonyl)glycylamino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine (127)**

N-(1,1-Dimethylethoxycarbonyl)glycine 95.58 g, 16.3 mmol) was added to 4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)benzeneamine (5.00 g, 8.2 mmol) in $CHCl_3$ (40 ml). The solution was heated at 40°C for 46 h. The solvent was evaporated. Chromatography ($CHCl_3$ / EtOAc 10:1) gave 5-(4-(N-(1,1-dimethylethoxycarbonyl)glycylamino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine (6.00 g, 95%) as a deep purple glass.

$^1$H NMR ($CDCl_3$)

δ 1.56 (9 H, s, Bu-t), 4.08 (1 H, s, Gly-$H_2$), 4.11 (1 H, s, Gly-$H_2$), 5.48 (1 H, br, Gly-NH), 7.73 (9 H, m, 3 × Ph 3,4,5-$H_3$), 7.92 (2 H, d, $J$ = 8.4 Hz, disubstituted aromatic-$H_2$), 8.18 (8 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic-$H_2$), 8.83 (6 H, s, *2,3,7,8,12,13,17,18*-$H_6$), 8.85 (2 H, t, $J$ = 4.8 Hz, *2,3,7,8,12,13,17,18*-$H_2$)

T.L.C. (C) Rf = 0.88

**5-(4-Glycylaminophenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine trihydrochloride (128)**

Hydrogen chloride was passed through 5-(4-(N-(1,1-dimethylethoxycarbonyl)glycyl-amino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine (2.24 g, 2.9 mmol) in $CH_2Cl_2$ (30 ml) for 1 h. The solvent and excess reagent were evaporated to reveal 5-(4-glycyl-aminophenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine trihydrochloride (2.27 g, quantitative) as an emerald green glass.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.34 (2 H, br, *21,22,23,24*-$H_4$), 0.45 (2 H, br, *21,22,2,24*-$H_4$), 4.07 (2 H, m, Gly-$H_2$), 7.86-8.84 (31 H, m, 3 × Ph 2,3,4,5,6-$H_5$ + disubstituted aromatic-$H_4$ + *2,3,7,8,12,13,17,18*-$H_8$ + NH + $NH_3^+$)

T.L.C. (C) Rf = 0.28

**N-(1,1-Dimethylethoxycarbonyl)sarcosine (151)**

Di-*t*-butyl dicarbonate (52.1 g, 239 mmol) in dioxane (200 ml) was added to sarcosine (20.0 g, 225 mmol) in water (100 ml) containing sodium hydroxide (12.7 g, 318.4 mmol) and stirred for 12 h. Water (200 ml) was then added and stirring continued for 6 h. The reaction was washed with $Et_2O$ (300 ml). EtOAc (300 ml) was added to the aqueous layer and the aqueous layer acidified by the addition of 10% aq. $H_2SO_4$. A white precipitate formed in the aqueous layer which slowly dissolved in the organic layer. When no more precipitate formed, the EtOAc layer was dried and the solvent was evaporated to give N-(1,1-dimethylethoxycarbonyl)sarcosine (35.53g, 84%) as a white crystalline solid. Mp 87-89°C (Lit. [296] mp. 89-90°C)

$^1$H NMR ($CDCl_3$)

δ 1.34 (9 H, s, Bu-t), 2.78 (2 H, s, Sar-$H_3$), 3.83 (2 H, s, Sar-$H_2$)

δ 1.39 (9 H, s, Bu-t), 2.82 (2 H, s, Sar-$H_3$), 3.84 (2 H, s, Sar-$H_2$)

T.L.C. (C) Rf = 0.37

**N-(1,1-Dimethylethoxycarbonyl)sarcosine 4-nitrophenyl ester (152)**

4-Nitrophenol (1.47 g, 10.58 mmol) was added to a stirred solution of N-(1,1-dimethyl-ethoxycarbonyl)sarcosine (2 g, 10.6 mmol) in dry dioxane (30 ml). After 15 min, DCC (2.18 g, 10.6 mmol) in dry dioxane (10 ml) was added to the stirred solution. Stirring was continued for 2 h at ambient temperature and the mixture allowed to stand at 4°C for

16 h. The suspension was allowed to warm to ambient temperature and was then filtered. The solvent was evaporated from the filtrate to give N-(1,1-dimethylethoxycarbonyl)-sarcosine 4-nitrophenyl ester (2.82 g, 64.5%) as an pale orange oil.

$^1$H NMR ($CDCl_3$)

δ 1.47 (9 H, s, Bu-t), 3.04 (3 H, s, Sar-$H_3$), 4.19 (2 H, s, Sar-$H_2$), 7.31 (1 H, d, $J$ = 8.8 Hz, Ar-2,6), 7.33 (1 H, d, $J$ = 8.8 Hz, Ar-2,6), 8.27 (1 H, d, $J$ = 9.4 Hz, Ar-3,5), 8.30 (1 H, d, $J$ = 9.4 Hz, Ar-3,5)

δ 1.49 (9 H, s, Bu-t), 3.02 (3 H, s, Sar-$H_3$), 4.26 (2 H, s, Sar-$H_2$), 7.31 (1 H, d, $J$ = 8.8 Hz, Ar-2,6), 7.33 (1 H, d, $J$ = 8.8 Hz, Ar-2,6), 8.27 (1 H, d, $J$ = 9.4 Hz, Ar-3,5), 8.30 (1 H, d, $J$ = 9.4 Hz, Ar-3,5)

T.L.C. (C) Rf = 0.95

MS (CI) 311 (M + H).

**N-(1,1-Dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (153)**

Ethane-1,2-diamine (12.0 g, 200 mmol) in $CH_2Cl_2$ (250 ml) was added dropwise over 30 min to a stirred solution of N-(1,1-dimethylethoxycarbonyl)sarcosine 4-nitrophenyl ester (2.96 g, 10 mmol) in $CH_2Cl_2$ (50 ml). After 2 h, the reaction mixture was washed once with water (50 ml) and twice with 10% aq. $Na_2CO_3$ (50 ml). The $CH_2Cl_2$ layer was dried with $Na_2CO_3$ and the solvent was evaporated to give N-(1,1-dimethylethoxy-carbonyl)sarcosine N-(2-aminoethyl)amide (2.27 g, 93%) as a pale orange oil.

$^1$H NMR ($CDCl_3$)

δ 1.47 (9H, s, Bu-t), 1.92 (2 H, br, $NH_2$), 2.84 (2 H, t, $J$ = 5.8 Hz, C$\underline{H_2}$N$H_2$), 2.95 (3 H, s, Sar-$H_3$), 3.33 (2 H, q, $J$ = 5.8 Hz, C$\underline{H_2}$NH), 3.87 (2 H , s, Ar-$CH_2$), 6.68 (1 H, br, NH)

T.L.C. (D) Rf = 0.28

MS (CI) 232 (M + H)

**Fluoren-9-ylmethyl pentafluorophenyl carbonate (156)**

Fluoren-9-ylmethyl chloroformate (2.00 g, 7.7 mmol) and pentafluorophenol (1.42 g, 7.7 mmol) were dissolved in $Et_2O$ (50 ml) and cooled to 0°C. Triethylamine (1.1 ml) was added dropwise to the stirred solution and stirring continued for 2 h. The solution was then washed three times with water (50 ml) and dried. The solution was then treated with charcoal. The solvent was evaporated to give fluoren-9-ylmethyl pentafluorophenyl carbonate (2.71 g, 87%) as a white solid. Mp 72 - 74°C (Lit. [270] mp. 84 - 86°C).

T.L.C. (C) Rf = 0.95

**N-(Phenylmethoxycarbonyl)sarcosine (158)**

Phenylmethyl chloroformate (27.0 g, 158 mmol) was added slowly to sarcosine (11.74 g, 132 mmol) in water (20 ml) containing NaOH (10.52 g, 264 mmol) and the mixture was stirred vigorously. After 5 h, the solution was washed with $Et_2O$ (20 ml) then acidified by addition of 10% aq. $H_2SO_4$ and extracted into EtOAc. The EtOAc layer was then washed with brine and dried. The solvent was evaporated to give N-(phenylmethoxycarbonyl)-sarcosine (27.98 g, 95%) as a colourless oil.

$^1H$ NMR ($CDCl_3$)

δ 2.99 (3 H, s, $CH_3$), 4.03 (2 H, s, Sar-$H_2$), 5.13 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 8.70 (1 H, br, OH)

δ 2.99 (3 H, s, $CH_3$), 4.08 (2 H, s, Sar-$H_2$), 5.16 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 8.70 (1 H, br, OH)

T.L.C. (C) Rf = 0.29

**N-(Phenylmethoxycarbonyl)sarcosine 4-nitrophenyl ester (159)**

4-Nitrophenol (1.53 g, 11 mmol) was added to a stirred solution of N-(phenylmethoxycarbonyl)sarcosine (2.47 g, 11 mmol) in dry dioxane (20 ml). After 15 min, DCC (2.27 g, 11 mmol) was added and the solution stirred for 2 h. The suspension was allowed to stand at 4°C for 16 h, then warmed to room temperature before being filtered. The solvent was evaporated from the filtrate to give N-(phenylmethoxycarbonyl)sarcosine 4-nitrophenyl ester (3.81 g, quantitative) as a yellow oil.

$^1$H NMR ($CDCl_3$)

δ 3.11 (3 H, s, Sar-$H_3$), 4.33 (2 H, s, Sar-$H_2$), 5.19 (2 H, s, Ar-$CH_2$), 7.35 (7 H, m, Ar + Ar'-2,6), 8.26 (2 H, m, Ar'-3,5)

δ 3.12 (3 H, s, Sar-$H_3$), 4.27 (2 H, s, Sar-$H_2$), 5.18 (2 H, s, Ar-$CH_2$), 7.35 (7 H, m, Ar + Ar'-2,6), 8.26 (2 H, m, Ar'-3,5)

T.L.C. (C) Rf = 0.90

**N-(Phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (160)**

Ethane-1,2-diamine (18.36 g, 306 mmol) in $CH_2Cl_2$ (300 ml) was added dropwise during 30 min to a stirred solution of N-(phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (6.16 g, 15.3 mmol) in $CH_2Cl_2$ (50 ml). The solution was stirred overnight and then washed once with water (50 ml) and twice with 10% aq. $Na_2CO_3$ (20 ml). The solution was dried and the solvent was evaporated to give N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (4.01 g, 99%) as a yellow oil.

This compound was also prepared using N-(phenylmethoxycarbonyl)sarcosine 4-nitrophenyl ester and using N-(phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester.

$^1$H NMR ($CDCl_3$)

δ 1.81 (2 H, br, $NH_2$) 2.75 (2 H, m, C$\underline{H_2}$-$NH_2$), 3.01 (3 H, s, $CH_3$), 3.26 (2 H, m, C$\underline{H_2}$-NH), 3.91 (2 H, s, Sar-$H_2$), 5.14 (2 H, s, Ar-$CH_2$), 6.42 (1 H, br, NH), 7.34 (5 H, m, Ar)

T.L.C. (E) Rf = 0.21

MS (CI) 266 (M+H).

**N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-pentafluorophenyl ester γ-phenylmethyl ester (162)**

N-(1,1-Dimethylethoxycarbonyl)glutamic acid γ-phenylmethyl ester (1.00 g, 2.9 mmol) in dry dioxane (10 ml) was cooled to 0°C. DCC (0.50g, 2.9 mmol) and pentafluorophenol (0.53 g, 2.9 mmol) were added to the stirred solution. The resulting suspension was stirred for 1 h at 0°C and 1 h at ambient temperature. The suspension was then filtered and the solvent was evaporated from the filtrate to give N-(1,1-dimethylethoxycarbonyl)-glutamic acid α-pentafluorophenyl ester γ-phenylmethyl ester (1.4 g, 96%) as a white solid. Mp 78 - 80°C.

$^1$H NMR ($CDCl_3$)

δ 1.45 (9 H, s, Bu-t), 1.21-1.47 (4 H, m, Glu β,γ-$H_2$), 4,70 (1 H, m, Glu α-H), 5.15 (2 H, s, Ar-$CH_2$), 5.18 (1 H, m, NH), 7.36 (5 H, s, Ar)

$^{19}$F NMR ($CDCl_3$)

δ -153.1 (2 F, d, *J* = 20 Hz, 2,6-Ar), -159.2 (1 F, t, *J* = 20 Hz, 4-Ar), -162.9 (2 F, t, *J* = 20 Hz, 3,5-Ar)

T.L.C. (F) Rf = 0.88

**N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-(N-(2-(N-(phenylmethoxy-carbonyl)sarcosyl)amino)ethyl)amide γ-phenylmethyl ester (163)**

N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-pentafluorophenyl ester γ-phenylmethyl ester (0.30 g, 1.1 mmol) was stirred with N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (0.569 g, 1.1 mmol) and N,N-diisopropylethylamine (0.16 g, 1.24 mmol) in $CH_2Cl_2$ (30 ml) for 20 h. The reaction mixture was washed once with cold

10% aq. $H_2SO_4$ (10 ml) and once with saturated aq. $NaHCO_3$ (10 ml). The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 10:1) gave N-(1,1-dimethylethoxycarbonyl)glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)-sarcosyl)amino)ethyl)amide γ-phenylmethyl ester (440 mg, 75%) as buff crystals. Mp 134-136°C.

$^1$H NMR ($CDCl_3$)

δ 1.42 (9 H, s, Bu-t), 1.91 (2 H, m, Glu β-$H_2$), 2.11 (2 H, m, Glu γ-$H_2$), 3.00 (3 H, s, Sar-$H_3$), 3.35 (4 H, br, $NCH_2CH_2N$), 3.89 (3 H, s, Sar-$H_2$), 4.10 (1 H, m, Glu α-H), 5.11 (2 H, s, Ar-$CH_2$), 5.16 (2 H, s, Ar-$CH_2$), 5.47 (1 H, m, NH), 6.76 (2 H, br, 2 × NH), 7.34 (10 H, m, Ar)

δ 1.42 (9 H, s, Bu-t), 1.91 (2 H, m, Glu β-$H_2$), 2.11 (2 H, m, Glu γ-$H_2$), 3.00 (3 H, s, Sar-$H_3$), 3.35 (4 H, br, $NCH_2CH_2N$), 3.90 (3 H, s, Sar-$H_2$), 4.10 (1 H, m, Glu α-H), 5.11 (2 H, s, Ar-$CH_2$), 5.16 (2 H, s, Ar-$CH_2$), 5.47 (1 H, m, NH), 6.76 (2 H, br, 2 × NH), 7.34 (10 H, m, Ar)

T.L.C. (C) Rf = 0.63

**Glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)sarcosyl)amino)ethyl)amide γ-phenylmethyl ester hydrochloride (164)**

Hydrogen chloride was bubbled through a solution of N-(1,1-dimethylethoxycarbonyl)-glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)sarcosyl)amino)ethyl)amide in $CH_2Cl_2$ for 30 min. The solvent and excess hydrogen chloride were evaporated to give glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)sarcosyl)amino)ethyl)amide γ-phenylmethyl ester hydrochloride (330m g, 85%) as an off-white glass.

$^1$H NMR ($(CD_3)_2SO$)

δ 2.34 (2 H, m, Glu β-$H_2$), 2.58 (2 H, m, Glu γ-$H_2$), 2.96 (3 H, s, Sar-$H_3$), 3.06-3.57 (4 H, br, $NCH_2CH_2N$), 3.92 (2 H, br, Sar-$H_2$), 4.24 (1 H, m, Glu α-H), 5.01 (2 H, s, Ar-$CH_2O_2CN$), 5.24 (2 H, s, Ar-$C\underline{H}_2O_2CCH$), 7.29 (10 H, m, Ar), 7.69 (1 H, br, NH), 8.38 (3 H, br, $NH_3^+$), 8.44 (1 H, m, NH)

δ 2.34 (2 H, m, Glu β-$H_2$), 2.58 (2 H, m, Glu γ-$H_2$), 2.96 (3 H, s, Sar-$H_3$), 3.06-3.57 (4 H, br, $NCH_2CH_2N$), 3.92 (2 H, br, Sar-$H_2$), 4.24 (1 H, m, Glu α-H), 5.04 (2 H, s, Ar-$CH_2O_2CN$), 5.24 (2 H, s, Ar-$C\underline{H}_2O_2CCH$), 7.29 (10 H, m, Ar), 7.69 (1 H, br, NH), 8.38 (3 H, br, $NH_3^+$), 8.44 (1 H, m, NH)

T.L.C. (C) Rf = 0.92

**N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (165)**

Pentafluorophenol (2.46 g, 13.45 mmol) in EtOAc (10 ml) was cooled to 0°C. DCC (2.32 g, 13.45 mmol) and N-(phenylmethoxycarbonyl)sarcosine (3.0 g, 13.45 mmol) in EtOAc (10 ml) were cooled to 0°C and added to the stirred solution of pentafluorophenol. The suspension was stirred at 0°C for 2 h then filtered. The solvent was evaporated from the filtrate and the residue redissolved in EtOAc. This was filtered and the solvent was removed from the filtrate to give N-(phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (4.66 g, 89%) as a pale pink oil.

IR (film) ν 1810 (ester C=O), 1715 (carbamate C=O).

$^1$H NMR ($CDCl_3$)

δ 3.08 (3 H, s, Sar-$H_3$), 4.43 (2 H, s, Sar-$H_2$), 5.19 (2 H, s, Ar-$CH_2$), 7.34 (5 H, s, Ar)

δ 3.09 (3 H, s, Sar-$H_3$), 4.36 (2 H, s, Sar-$H_2$), 5.16 (2 H, s, Ar-$CH_2$), 7.36 (5 H, s, Ar)

$^{19}$F NMR ($CDCl_3$)

δ -152.6 (2 F, d, $J$ = 23 Hz, 2,6-Ar), -157.6 (1 F, t, $J$ = 21 Hz, 4-Ar), -162.3 (2 F, dd, $J$ = 21, 23 Hz, 3,5-Ar)

δ -153.1 (2 F, d, $J$ = 23 Hz, 2,6-Ar), -157.8 (1 F, t, $J$ = 21 Hz, 4-Ar), -162.4 (2 F, dd, $J$ = 21, 23 Hz, 3,5-Ar)

T.L.C. (G) Rf = 0.56

**N-(N-(Phenylmethoxycarbonyl)sarcosyl)glutamic acid α-(N-(2-(N-(phenylmethoxy-carbonyl)sarcosyl)amino)ethyl)amide γ-phenylmethyl ester (166)**

Glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)sarcosyl)amino)ethyl)amide γ-phenylmethyl ester hydrochloride (320 mg, 0.62 mmol) was stirred with N-(phenyl-methoxycarbonyl)sarcosine pentafluorophenol ester (239 mg, 0.62 mmol) and N,N-diisopropylethylamine (130 mg, 1.24 mmol) in $CH_2Cl_2$ for 20 h. The reaction mixture was washed once with water (10 ml), twice with 10% aq. $H_2SO_4$ (10 ml), once with saturated aq. $NaHCO_3$ (10 ml) and once with brine. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 10:1) gave N-(N-(phenylmethoxy-carbonyl)sarcosyl)glutamic acid α-(N-(2-(N-(phenylmethoxycarbonyl)sarcosyl)amino)-ethyl)amide γ-phenylmethyl ester (260 mg, 73%) as a viscous straw-coloured oil.

$^1$H NMR ($CDCl_3$)

δ 1.95 (1 H, m, Glu β-$H_2$), 2.14 (1 H, m, Glu β-$H_2$), 2.46 (2 H, Glu γ-$H_2$), 3.02 (3 H, s, Sar-$H_3$), 3.31 (4 H, br, $NCH_2CH_2N$), 3.79 (2 H, s, Sar-$H_2$), 3.89 (2 H, br, Sar-$H_2$), 4.36 (1 H, m, Glu α-H), 5.13 (6 H, m, 2 × Ar-$CH_2$ + Ar'-$CH_2$), 6.61 (1 H, br, NH), 6.82 (1 H, br, NH), 7.00(1 H, br, NH), 7.34 (15 H, br, Ar + Ar')

T.L.C. (C) Rf = 0.42

**N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-phenylmethyl ester γ-(2,4,5-tri-chlorophenyl) ester (168)**

N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-phenylmethyl ester (24.86 g, 73.7 mmol) was stirred with DCC (15.2 g, 73.7 mmol) and 2,4,5-trichlorophenol (14.4 g, 73.7 mmol) in EtOAc (300 ml) at 0°C for 18 h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc, filtered and the solvent was evaporated to give N-(1,1-dimethylethoxycarbonyl)glutamic acid α-

phenylmethyl ester γ-(2,4,5-trichlorophenyl) ester (37.6 g, 98%) as an off-white solid. Mp. 78-80°C

$^1$H NMR ($CDCl_3$)

δ 1.45 (9 H, s, Bu-t), 2.34 (2 H, Glu β-$H_2$), 2.69 (2 H, m, Glu γ-$H_2$), 4.46 (1 H, m, Glu α-H), 5.19 (3 H, m, Ar-$CH_2$ + NH), 7.29 (1 H, s, Ar'-6H), 7.36 (5 H, s, Ar), 7.53 (1 H, s, Ar'-3H)

T.L.C. (C) Rf = 0.79

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine phenylmethyl ester (169)**

N-(1,1-Dimethylethoxycarbonyl)glutamic acid α-phenylmethyl ester γ-(2,4,5-trichlorophenyl) ester (8.22 g, 14.8 mmol) was added to a solution of methyl 6-aminohexanoate hydrochloride (2.79 g, 14.8 mmol), N,N-diisopropylethylamine (4.18 g, 29,6 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (50 ml). The mixture was stirred for 4 d and the solvent was evaporated. The residue was dissolved in EtOAc and washed with cold 10% aq. $H_2SO_4$, and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 40 : 1) gave $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine phenylmethyl ester (5.93 g, 87 %) as a white solid. Mp 60-63°C

$^1$H NMR ($CDCl_3$)

δ 1.28-1.51 (13 H, m, Bu-t + $NCH_2C\underline{H_2}C\underline{H_2}CH_2CH_2$), 1.63 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.90 (2 H, m, Glu β-$H_2$), 2.22 (2 H, m, Glu γ-$H_2$), 2.31 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.23 (2 H, q, $J$ = 6.6 Hz, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.66 (3 H, s, $CH_3$), 4.30 (1 H, m, Glu α-H), 5.14 (1 H, s, Ar-$CH_2$), 5.18 (1 H, s, Ar-$CH_2$), 5.39 (1 H, d, $J$ = 9 Hz, α-NH), 6.23 (1 H, br, $N\underline{H}CH_2$), 7.35 (5 H, s, Ar)

MS (FAB+) 465 (M + H)

T.L.C. (C) Rf = 0.33

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine (170)**

10% Palladium on charcoal (2.2 g) was added to a solution of $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine phenylmethyl ester (11.97 g, 25.8 mmol) in THF (150 ml). The mixture was treated with hydrogen for 24 h then filtered through Celite®. The solvent was evaporated from the filtrate to give $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine (8.81 g, 91%) as a pale green oil.

$^1$H NMR ($CDCl_3$)

δ 1.24-1.63 (15 H, m, Bu-t + $NCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2$), 1.84 (2 H, m, Glu β-$H_2$), 1.91-2.15 (2 H, m, Glu γ-$H_2$), 2.32 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 3.28 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.67 (3 H, s, $OCH_3$), 4.22 (1 H, m, Glu α-H), 5.61 (1 H, br, $N\underline{H}CH_2$)

T.L.C. (C) Rf = 0.08

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine 2,4,5-trichlorophenyl ester (171)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine (27.0 g, 72 mmol) was added to a stirred solution of DCC (14.86 g, 72 mmol) and 2,4,5-trichlorophenol (14.24 g, 72 mmol) in EtOAc (500 ml). The resulting suspension was stirred for 18 h and was then filtered. The filtrate was evaporated and the residue redissolved in EtOAc and filtered. The solvent was evaporated to give an oil which, on trituration with $Et_2O$, yielded $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine 2,4,5 trichlorophenyl ester (28.04 g, 70%) as a white solid. Mp 92-94°C.

$^1$H NMR ($CDCl_3$)

δ 1.36-1.50 (13 H, m, Bu-t + $NCH_2C\underline{H}_2C\underline{H}_2CH_2CH_2$), 1.75 ($NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.91 (2 H, m, Glu β-$H_2$), 2.14 (2 H, m, Glu γ-$H_2$), 2.6 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 3.49 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.91 (3 H, s, $OCH_3$), 4.42 (1 H, m, Glu α-H),

5.59 (1 H, m, N<u>H</u>$CH_2$), 6.48 (1 H, m, Glu-NH), 7.36 (1 H, s, Ar-6H), 7.64 (1 H, s, Ar-3H)

T.L.C. (C) Rf = 0.80

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (172)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine 2,4,5-trichlorophenyl ester (11.09 g, 20 mmol) was added to a stirred solution of N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (5.30 g, 20 mmol) in $CH_2Cl_2$ (50 ml) containing N,N-diisopropylethylamine (5.69 g, 44 mmol) and DMAP (10 mg). The solution was stirred for 4 d, then washed twice with cold 10% aq. $H_2SO_4$ and twice with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated to give an oil. Trituration with $Et_2O$ yielded $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (8.97 g, 72%) as a buff solid. Mp 88-90°C

$^1$H NMR ($(CD_3)_2SO$)

δ 1.43 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.47 (11 H, m, Bu-t + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.51 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.60 (2 H, m, Glu β-$H_2$), 2.00 (2 H, br, Glu γ-$H_2$), 2.29 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 3.01 (3 H, s, Sar-$H_3$), 3.18 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.36 (4 H, br, $NCH_2CH_2N$), 3.63 (3 H, $OCH_3$), 3.89 (2 H, s, Sar-$H_2$), 4.12 (1 H, m, Glu α-H), 5.13 (2 H, s, Ar-$CH_2$), 5.85 (1 H, m, NH), 6.46 (1 H, m, NH), 7.11 (1 H, br, NH), 7.34 (6 H, m, Ar + NH)

T.L.C. (C) Rf = 0.43

**$N^{\delta}$-(6-Methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (173)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide (8.97 g, 14.4 mmol) in $CH_2Cl_2$ (300 ml) for 30 min. MeOH (1 ml) was added and the solvents were evaporated to give $N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (7.96 g, 99%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.43 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.47 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.51 (4 H, m, Glu β-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.92 (2 H, br, Glu γ-$H_2$), 2.21 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.85 (3 H, s, Sar-$H_3$), 3.02 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.16 (4 H, br, $NCH_2CH_2N$), 3.50 (3 H, $OCH_3$), 3.86 (2 H, s, Sar-$H_2$), 4.12 (1 H, m, Glu α-H), 5.04 (2 H, s, Ar-$CH_2$), 7.32 (5 H, m, Ar), 8.03 (1 H, br, NH), 8.19 (1 H, m, NH), 8.32 (3 H, m, $NH_3^+$), 8.70 (1 H, br, NH)

δ 1.43 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.47 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.51 (4 H, m, Glu β-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.92 (2 H, br, Glu γ-$H_2$), 2.21 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.89 (3 H, s, Sar-$H_3$), 3.02 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.16 (4 H, br, $NCH_2CH_2N$), 3.50 (3 H, $OCH_3$), 3.87 (2 H, s, Sar-$H_2$), 4.12 (1 H, m, Glu α-H), 5.07 (2 H, s, Ar-$CH_2$), 7.32 (5 H, m, Ar), 8.03 (1 H, br, NH), 8.19 (1 H, m, NH), 8.32 (3 H, m, $NH_3^+$), 8.70 (1 H, br, NH)

T.L.C. (C) Rf = 0.05

**$N^{\delta}$-(6-Methoxy-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (174)**

N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (5.33 g, 13.23 mmol) was added to $N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (2.46 g, 4.41 mmol), N,N-diisopropylethyl-

amine (2.28 g, 17.64 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (50 ml). The solution was stirred for 48 h. The solution was washed with cold 10% aq. $H_2SO_4$, with 10% aq. $Na_2CO_3$ and with brine. The solution was dried and the solvent evaporated. Chromatography ($CH_2Cl_2$ / MeOH 40:1 then $CH_2Cl_2$ / MeOH 30:1) gave $N^{\delta}$-(6-methoxy-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.23 g, 70%) as a pale yellow glass.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.24 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.72 (1 H, m, Glu β-$H_2$), 1.88 (1 H, m, Glu β-$H_2$), 2.26 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.85 (3 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 2.99 (2 H, q, $J$ = 6.3 Hz, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.83 (2 H, s, Sar-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Glu α-H), 5.03 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.36 (10 H, m, Ar), 7.77 (1 H, m, $N\underline{H}CH_2CH_2CH_2$), 7.90 (2 H, br, $N\underline{H}CH_2CH_2N\underline{H}$), 8.18 (1 H, d, $J$ = 6.8 Hz, Glu-NH)

δ 1.24 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.72 (1 H, m, Glu β-$H_2$), 1.88 (1 H, m, Glu β-$H_2$), 2.26 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.86 (3 H, s, Sar-$H_3$), 2.99 (2 H, q, $J$ = 6.3 Hz, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.00 (3 H, s, Sar-$H_3$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.83 (2 H, s, Sar-$H_2$), 3.93 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Glu α-H), 5.03 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.36 (10 H, m, Ar), 7.77 (1 H, m, $N\underline{H}CH_2CH_2CH_2$), 7.90 (2 H, br, $N\underline{H}CH_2CH_2N\underline{H}$), 8.18 (1 H, d, $J$ = 6.8 Hz, Glu-NH)

T.L.C. (C) Rf = 0.47

MS (FAB+) 727 (M + H).

**$N^{\varepsilon}$-Trifluoroacetyllysine (181)**

Trifluoroacetic anhydride (65.5 g, 313 mmol) was added dropwise during one hour to ethanethiol (12.42 g, 200 mmol) cooled in an ice-water bath. The stirred solution was allowed to stand under a reflux condenser fitted with a $CaCl_2$ drying tube for 1 h then

heated at reflux for 16 h. The solution was allowed to cool to room temperature and then dissolved in $CH_2Cl_2$. This was washed twice with 5% aqueous KOH (200 ml) then once with water and once with brine. The solution was dried and then distilled to give ethyl trifluorothioacetate (9.32 g, 49%) as a colourless liquid. The isolated compound (8 g, 50.6 mmol) was added to lysine monohydrochloride (5.86 g, 32 mmol) in 1M aqueous NaOH (32 ml). The mixture was stirred vigorously for 6 h and then cooled in an ice-water bath. A precipitate formed and was collected by filtration. The crude solid was recrystallised from water and ethanol to give crude $N^{\varepsilon}$-trifluoroacetyllysine (550 mg, 7.1%). The product was not soluble in any available NMR solvents.

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine (190)**

Lysine monohydrochloride (62.0g, 339 mmol) was boiled under reflux with $CuCO_3$ (121.7 g, 509 mmol) in water (1200 ml) for 4 h. The solution was filtered whilst hot and the filtrate was allowed to cool to 20°C. Trichloroethyl chloroformate (143.2 g, 679 mmol) and aqueous $Na_2CO_3$ (108 g, 1.02 mol) were added alternately in portions to the filtrate during 2 h. The solution was stirred vigorously at 0°C for 20 h. The precipitate was collected and was boiled under reflux with ethylenediaminetetraacetic acid disodium salt (126.3 g, 679 mmol) in water (2000 ml) for 2 h. The solution was cooled to 0°C during 48 h and the crude $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine was collected by filtration. Di-*t*-butyl dicarbonate (92.6 g, 424 mmol) in dioxane (200 ml) was added to this material in water (500 ml) and triethylamine (20.2 g, 200 mmol) and the mixture was stirred vigorously for 4 d. The mixture was washed with $Et_2O$ (500 ml). EtOAc (500 ml) was added to the mixture which was then acidified by addition of cold 10% aq. $H_2SO_4$. The organic layer was dried and the solvent was evaporated to give $N^{\alpha}$-(1,1-dimethyl-ethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine (105.2 g, 75%) as a cream wax.

$^1$H NMR ($CDCl_3$)

δ 1.45 (9 H , s, Bu-t), 1.58-1.88 (6 H, m, Lys β,γ,δ-$H_2$), 3.23 (2 H, q, $J$ = 6.7 Hz, Lys ε-$H_2$), 4.30 (1 H, m, Lys α-H), 4.73 (2 H, s, $CH_2CCl_3$), 5.28 (1 H, m, NH), 6.38 (1H, m, NH)

T.L.C. (C) Rf = 0.43

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine pentafluorophenyl ester (191)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine (2.00 g, 4.7 mmol) was stirred with DCC (0.82 g, 4.7 mmol) and pentafluorophenol (0.87 g, 4.7 mmol) in EtOAc (60 ml) at 0°C for 6 h. The suspension was filtered and the solvent was evaporated from the filtrate to give $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine pentafluorophenyl ester (2.50 g, 91%) as a yellow oil.

IR (film) ν 3360 (N-H), 1800 (ester C=O), 1725 (carbamate C=O).

$^1$H NMR ($CDCl_3$)

δ 1.47 (9 H, s, Bu-t), 1.51-1.95 (6 H, m, Lys β,γ,δ-$H_2$), 3.28 (2 H, q, $J$ = 7.3 Hz, Lys ε–$H_2$), 4.61 (1 H, m, Lys α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.22 (2 H, m, 2 × NH)

$^{19}$F NMR ($CDCl_3$)

δ -152.7 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -158.6 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.7 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

T.L.C. (C) Rf = 0.75

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (192)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine pentafluorophenyl ester (2.0 g, 3.5 mmol) was stirred with N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (918 mg, 3.5 mmol) and N,N-diisopropylethylamine (490 mg, 3.8 mmol) in $CH_2Cl_2$ for 5 h. The solution was washed with cold 10% aq. $H_2SO_4$ (10 ml) and with saturated aq. $NaHCO_3$ (10 ml). The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 10:1) gave $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (1.97 g, 85%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.11-1.71 (15 H, m, Bu-t + Lys β,γ,δ-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 3.01 (2 H, q, $J$ = 6.7 Hz, Lys ε-$H_2$), 3.14 (4 H, m, $NCH_2CH_2N$), 3.82 (3 H, br, Sar-$H_2$ + Lys α-H), 4.76 (2 H, s, $CH_2CCl_3$), 5.05 (3 H, s, Ar-$H_2$), 6.74 (1 H, d, $J$ = 8.5 Hz, Lys NH), 7.36 (5 H, m, Ar), 7.64 (1 H, t, $J$ = 5.1 Hz, NH), 7.87 (1 H, br, NH), 7.94 (1 H, m, NH).

δ 1.11-1.71 (15 H, m, Bu-t + Lys β,γ,δ-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.01 (2 H, q, $J$ = 6.7 Hz, Lys ε-$H_2$), 3.14 (4 H, m, $NCH_2CH_2N$), 3.82 (3 H, br, Sar-$H_2$ + Lys α-H), 4.76 (2H, s, $CH_2CCl_3$), 5.08 (3 H, s, Ar-$H_2$), 6.74 (1 H, d, $J$ = 8.5 Hz, Lys NH), 7.36 (5 H, m, Ar), 7.64 (1 H, t, $J$ = 5.1 Hz, NH), 7.87 (1 H, br, NH), 7.94 (1 H, m, NH).

T.L.C. (C) Rf = 0.31

MS (FAB+) 670 (M + H).

**$N^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (193)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (1.64 g, 2.45 mmol) in $CH_2Cl_2$ (20 ml) for 30 min. The solvent and

excess reagent were evaporated to give $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.47 g, 99%) as a colourless glass.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.26 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.67 (2 H, m, Lys β,γ,δ–$H_2$), 2.84 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.66 (1 H, Lys α-H), 3.84 (2 H, s, Sar-$H_2$), 4.76 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 7.69 (1 H, m, NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.71 (1 H, m, NH)

δ 1.26 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.67 (2 H, m, Lys β,γ,δ-$H_2$), 2.87 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.66 (1 H, Lys α-H), 3.85 (2 H, s, Sar-$H_2$), 4.76 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 7.69 (1 H, m, NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.71 (1 H, m, NH)

T.L.C. (C) Rf = 0.08

MS (FAB-) 604 (M + Cl).

**$N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (194)**

$N^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (1.41 g, 2.3 mmol) was stirred with N-(phenylmethoxy-carbonyl)sarcosine pentafluorophenyl ester (908 mg, 2.3 mmol) and triethylamine (470 mg, 4.68 mmol) in $CH_2Cl_2$ (30 ml) for 5 h. The solution was washed with water (10 ml), with cold 10% aq. $H_2SO_4$ (10 ml) and with saturated aq. $NaHCO_3$ (10 ml). The solution was dried and the solvent was evaporated. The residue was redissolved in $CH_2Cl_2$ and washed twice with cold 10% aq. $H_2SO_4$ and four times with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 10:1) gave $N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.08 g, 60%) as a brown solid. Mp 76 - 78°C.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 1.24 (2 H, m, Lys β,γ-$H_2$), 1.38 (2 H, m, Lys δ-$H_2$), 1.62 (2 H, m, Lys β,γ-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.98 (2 H, m, Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.82 (2 H, s, Sar-$H_2$), 3.93 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Lys α-H), 4.79 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.34 (10 H, m, Ar), 7.68 (1 H, m, NH), 8.02 (3 H, m, 3 × NH).

δ 1.24 (2 H, m, Lys β,γ-$H_2$), 1.38 (2 H, m, Lys δ-$H_2$), 1.62 (2 H, m, Lys β,γ-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.98 (2 H, m, Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.82 (2 H, s, Sar-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Lys α-H), 4.79 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.34 (10 H, m, Ar), 7.68 (1 H, m, NH), 8.02 (3 H, m, 3 × NH).

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 1.25 (2 H, m, Lys β,γ,δ-$H_2$), 1.46 (2 H, m, Lys β,γ,δ-$H_2$), 1.65 (2 H, m, Lys β,γ,δ-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 3.02 (2 H, m, Lys ε-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.82 (2 H, s, Sar-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 4.22 (1 H, m, Lys α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (4 H, s, Ar-$CH_2$), 7.33 (11 H, m, Ar + NH), 7.66 (2 H, m, 2 × NH), 7.73 (1 H, m, NH).

T.L.C. (C) Rf = 0.43

MS (FAB+) 775 (M+ H).

**N-(1,1-Dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (197)**

N-(1,1-Dimethylethoxycarbonyl)glycine (16.6 g, 95 mmol) was stirred with DCC (19.57 g, 95 mmol) and 2,4,5-trichlorophenol (18.76 g, 95 mmol) in EtOAc (150 ml) at 0°C for 16 h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc and filtered. The solvent was evaporated from the filtrate to give N-(1,1-dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (32.2 g, 95%) as a white solid. Mp 95 - 97°C (Lit. mp. 106 - 107°C).

$^1$H NMR ($CDCl_3$)

δ 1.47 (9 H, s, Bu-t), 4.22 (2 H, d, $J$ = 5.9 Hz, Gly-$H_2$), 5.08 (1 H, br, NH), 7.32 (1 H, s, Ar-6H), 7.56 (1 H, s, Ar-3H)

T.L.C. (C) Rf = 0.81

**N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (198)**

N-(1,1-Dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (4.96 g, 14 mmol) was stirred with N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (3.71 g, 14 mmol) and N,N-diisopropylethylamine (1.99 g, 15.4 mmol) in $CH_2Cl_2$ (100 ml) for 20 h. The solution was washed twice with cold 10% aq. $H_2SO_4$ and four times with saturated aq. $NaHCO_3$. The solution was dried and the solvent was evaporated. Chromatography (EtOAc / MeOH 10:1, then EtOAc / MeOH 5:1, then EtOAc / MeOH 3 : 1) gave N-(1,1-dimethylethoxycarbonyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (4.53 g, 79%) as a colourless foam.

$^1$H NMR ($CDCl_3$)

δ 1.42 (9 H, s, Bu-t), 3.00 (3 H, s, Sar-$H_3$), 3.38 (4 H, br, $NCH_2CH_2N$), 3.74 (2 H, m, Gly-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 5.23 (2 H, s, Ar-$CH_2$), 5.49 (1 H, br, Gly-NH), 6.73 (2 H, br, $\underline{H}NCH_2CH_2N\underline{H}$), 7.38 (5 H, m, Ar)

T.L.C. (C) Rf = 0.41

MS (FAB+) 423 (M + H).

**Glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (199)**

Hydrogen chloride was bubbled through a solution of N-(1,1-dimethylethoxycarbonyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.04 g, 4.95 mmol) in $CH_2Cl_2$ (50 ml) for 1 h. The solvent and excess reagent were evaporated to give glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.71 g, 97%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 2.86 (3 H, s, $CH_3$), 3.15 (4 H, br, $NCH_2CH_2N$), 3.49 (2 H, m, Gly-$H_2$), 3.86 (2 H, s, Sar-$H_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 8.20 (3 H, m, $NH_3^+$) 8.63-9.12 (2 H, m, 2 × NH)

δ 2.89 (3 H, s, $CH_3$), 3.15 (4 H, m, $NCH_2CH_2N$), 3.51 (2 H, m, Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 5.04 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 8.20 (3 H, m, $NH_3^+$) 8.63-9.12 (2 H, m, 2 × NH)

T.L.C. (C) Rf = 0.10

MS (FAB+) 323 (M + H).

**N-(1,1-Dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester (200)**

N-(1,1-Dimethylethoxycarbonyl)leucine (3.52 g, 15.2 mmol) was stirred with DCC (3.14 g, 15.2 mmol) and 2,4,5-trichlorophenol (3.01 g, 15.2 mmol) in EtOAc (50 ml) at -10°C for 4 h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc and filtered. The solvent was evaporated from the filtrate to give N-(1,1-dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester (6.2 g, 99%) as a buff wax.

$^1$H NMR ($CDCl_3$)

δ 1.02 (6 H, d, $J$ = 6.4 Hz, 2 × Leu-$CH_3$), 1.47 (9 H, s, Bu-t), 1.65 (1 H, m, Leu β-$H_2$), 1.84 (2 H, m, Leu γ-H + Leu-β-$H_2$), 4.56 (1 H, dt, $J$ = 4, 8 Hz, Leu α-H), 4.92 (1 H, d, $J$ = 8 Hz, NH), 7.32 (1 H, s, Ar-6H), 7.55 (1 H, s, Ar-3H)

T.L.C. (C) Rf = 0.91

**N-(N-(1,1-Dimethylethoxycarbonyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (201)**

Glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (3.21 g, 7.9 mmol) was stirred with N-(1,1-dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester (3.19 g, 7.8 mmol) and N,N-diisopropylethylamine (3.21 g, 25 mmol) in DMF (30 ml) for 3 d. The solvent was evaporated and the residue was dissolved in EtOAc. The solution was washed twice with cold 10% aq. $H_2SO_4$ and twice with 10% aq. $Na_2CO_3$. The solvent was evaporated and the residue was dissolved in MeOH. NaOH (1 M, 8 ml, 8 mmol) was added and the solution stirred for 5 min. The MeOH was evaporated and the aqueous layer was extracted with EtOAc. The EtOAc layer was dried and the solvent was evaporated to give N-(N-(1,1-dimethylethoxycarbonyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.26 g, 78%) as a pale brown oil.

$^1$H NMR ($CDCl_3$)

δ 0.86 (6 H, d, $J$ = 5.8 Hz, 2 × $CH_3$), 1.36 (10 H, s, Bu-t + Leu β-$H_2$), 1.61 (2 H, br, Leu β-$H_2$ + Leu γ-H), 3.03 (3 H, s, Sar-$H_3$), 3.26 (4 H, br, $NCH_2CH_2N$), 3.74 (2 H, m, Gly-$H_2$), 4.02 (3 H, m, Sar-$H_2$ + Leu α-H), 5.07 (2 H, s, Ar-$CH_2$), 5.16 (1 H, m, NH), 6.89 (1 H, m, NH), 7.16 (1 H, m, NH), 7.40 (6 H, m, Ar + NH)

T.L.C. (C) Rf = 0.48

MS (FAB-) 534 (M - H)

**N-Leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (202)**

Hydrogen chloride was bubbled through a solution of N-(1,1-dimethylethoxycarbonyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl amide (3.00 g, 5.61 mmol) in $CH_2Cl_2$ (50 ml) for 1 h. The solvent and excess hydrogen chloride were evaporated to give N-leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide hydrochloride (2.6 g, quantitative) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 0.90 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 1.52-1.60 (2 H, m, Leu β-$H_2$), 1.62-1.70 (1 H, m, Leu γ-H), 2.85 (3 H, s, Sar-$CH_3$), 3.13 (4 H, br, $NCH_2CH_2N$), 3.67 (1 H, dd, $J$ = 16, 5 Hz, Gly-$H_2$), 3.76-3.86 (4 H, s, Sar-$H_2$ + Gly-$H_2$ + Leu α-H), 5.04 (2 H, s, Ar-$CH_2$), 7.33 (5 H, m, Ar), 8.12- 8.19 (2 H, br, 2 × NH), 8.42 (3 H, br, $NH_3^+$), 8.98 (1 H, t, $J$ = 5.9 Hz, NH)

δ 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 0.90 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 1.52-1.60 (2 H, m, Leu β-$H_2$), 1.62-1.70 (1 H, m, Leu γ-H), 2.88 (3 H, s, Sar-$CH_3$), 3.13 (4 H, m, $NCH_2CH_2N$), 3.67 (1 H, dd, $J$ = 16, 5 Hz, Gly-$H_2$), 3.85 (4 H, s, Sar-$H_2$ + Gly-$H_2$ + Leu α-H), 5.07 (2 H, s, Ar-$CH_2$), 7.33 (5 H, m, Ar), 8.12-8.19 (1 H, br, 2 × NH), 8.42 (3 H, br, $NH_3^+$), 8.98 (1 H, t, $J$ = 5.9 Hz, NH).

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.92 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.52-1.72 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.91 (3 H, s, Sar-$H_3$), 3.13 (4 H, br, $NCH_2CH_2N$), 3.62 (2 H, m, Gly-$H_2$), 3.89 (3 H, m, Sar-$H_2$ + Leu α-H), 5.08 (2 H, s, Ar-$CH_2$), 7.39 (5 H, Ar), 7.91 (1 H, br, NH), 7.96 (1 H, br, NH), 8.38 (3 H, br, $NH_3^+$), 8.67 (1 H, m, NH)

T.L.C. (C) Rf = 0.09

MS (FAB+) 436 (M + H).

Acc. Mass 436.2551 (M + H) ($C_{21}H_{34}N_5O_5$ requires 436.2560)

**N-(1,1-Dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (203)**

N-(1,1-Dimethylethoxycarbonyl)phenylalanine (6.36 g, 24 mmol) was stirred with DCC (4.94 g, 24 mmol) and pentafluorophenol (4.42 g, 24 mmol) in EtOAc at 0°C for 3 h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc and filtered to give N-(1,1-dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (10.32 g, 99%) as a white solid. Mp 99 - 101°C (Lit. [297] mp. 111-112°C)

$^1$H NMR ($CDCl_3$)

δ 1.43 (9 H, s, Bu-t), 3.20 (1 H, dd, $J$ = 14.0, 6.1 Hz, Phe β-$H_2$), 3.31 (1 H, dd, $J$ = 14.0, 5.3 Hz, Phe β-$H_2$), 4.75 (1 H, m, Phe α-H), 4.93 (1H, br, NH), 7.32 (5 H, m, Ar)

$^{19}$F NMR ($CDCl_3$)

δ -152.6 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -158.5 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.9 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

δ -153.3 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -157.5 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.4 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

T.L.C. (C) Rf = 0.92

**N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (204)**

N-Leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.09 g, 2.4 mmol) was added to a stirred solution of N-(1,1-dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (948 mg, 2.4 mmol) and N,N-diisopropylethylamine (678 mg, 5.3 mmol) in $CH_2Cl_2$ (30 ml) and the mixture was stirred for 16 h. The solution was washed with cold 10% aq. $H_2SO_4$ (10 ml), with 10% aq. $Na_2CO_3$ (10 ml) and with brine (10 ml). The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ then $CHCl_3$ / MeOH 20 : 1) gave N-(N-(N-(1,1-dimethyl-

ethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (1.05 g, 65%) as a buff foam.

$^1$H NMR ($CDCl_3$)

δ 0.89 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 0.91 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 1.40 (9 H, s, Bu-t), 1,49 (2 H, m, Leu β-$H_2$), 1.71 (1 H, m, Leu γ-H), 2.93 (1 H, m, Phe β-$H_2$), 3.03 (3 H, m, Sar-$CH_3$), 3.32 (5 H, m, $NCH_2CH_2N$+ Phe β-$H_2$), 3.79 (2H, m, Gly-$H_2$), 3.94 (2 H, s, Sar-$H_2$), 4.29 (2 H, m, Phe α-H + Leu α-H), 5.14 (2 H, s, Ar-$CH_2$), 5.44 (1 H, m, NH), 6.86 (1 H, m, NH), 6.96 (1 H, m, NH), 7.07 (1 H, m, NH), 7.27 (11 H, m, Ar + NH)

T.L.C. (C) Rf = 0.38

MS (FAB +) 683 (M +H).

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester (205)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine (6.32 g, 15 mmol) was stirred with DCC (3.10 g,15 mmol) and 2,4,5-trichlorophenol (2.96 g, 15 mmol) in EtOAc (100 ml) at 0°C for 20 h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc and filtered. The solvent was evaporated from the filtrate to give $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester (8.04 g, 99%) as a viscous yellow oil.

$^1$H NMR ($CDCl_3$)

δ 1.47 (9 H, s, Bu-t), 1.52- 1.96 (6 H, m, Lys β,γ,δ-$H_2$), 3.27 (2 H, q, $J$ = 6.4 Hz, Lys ε-$H_2$), 4.54 (1 H, m, Lys α-H), 4.73 (2 H, s, $CH_2CCl_3$), 5.18 (2 H, m, 2 × NH), 7.32 (1 H, s, Ar-6H), 7.56 (1 H, s, Ar-3H)

T.L.C. (C) Rf = 0.89

**N-(N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)-glycine methyl ester (206)**

N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester (7.8 g, 14.5 mmol) was stirred with glycine methyl ester hydrochloride (1.82 g, 14.5 mmol) and N,N-diisopropylethylamine (4.7 g, 36 mmol) in $CH_2Cl_2$ (50 ml) for 20 h. The solution was washed with cold 10% aq. $H_2SO_4$ (50 ml), with 10% aq. $Na_2CO_3$, with water and with brine. The solution was dried and the solvent was evaporated. The residue was dissolved in $CH_2Cl_2$ and washed twice with cold 10% aq. $H_2SO_4$ and twice with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ then $CHCl_3$ / MeOH 20:1 then $CHCl_3$ / MeOH 15 : 1) gave N-(N$^{\alpha}$-(1,1-dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysyl)glycine methyl ester (5.7 g, 82%) as a yellow oil.

$^1$H NMR ($CDCl_3$)

δ 1.18-1.77 (13 H, m, Bu-t + Lys β,γ-$H_2$), 1.95 (2 H, m, Lys δ-$H_2$), 3.30 (2 H, q, $J$ = 6.4 Hz, Lys ε-$H_2$), 3.82 (3 H, s, $OCH_3$), 4.10 (2 H, t, $J$ = 6.2 Hz, Gly-$H_2$), 4.20 (1 H, m, Lys α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.25 (1 H, d, $J$ = 7.9 Hz, Lys-NH), 5.37 (1 H, t, $J$ = 4.5 Hz, NH), 6.83 (1 H, m, NH)

T.L.C. (C) Rf = 0.70

**N-(N$^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysyl)glycine methyl ester hydrochloride (207)**

Hydrogen chloride was bubbled through a solution of N-(N$^{\alpha}$-(1,1-dimethylethoxy-carbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycine methyl ester (5.65 g, 11.8 mmol) in $CH_2Cl_2$ (50 ml) for 30 min. The solvent and excess hydrogen chloride were evaporated to give N-(N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycine methyl ester hydrochloride (4.66 g, 95%) as a pale yellow oil.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.25 (4 H, m, Lys β + γ-$H_2$), 1.74 (2 H, m, Lys δ-$H_2$), 3.01 (2 H, q, $J$ = 6.4 Hz, Lys ε–$H_2$), 3.65 (3 H, s, $OCH_3$), 3.82 (1 H, m, Lys α-H), 3.91 (1 H, dd, $J$ = 17.6, 5.9 Hz, Gly-$H_2$), 3.98 (1 H, dd, $J$ = 17.6, 5.9 Hz, Gly-$H_2$), 4.79 (2 H, s, $CH_2CCl_3$), 7.68 (1 H, t, $J$ = 5.4 Hz, NH), 8.31 (3 H, br, $NH_3^+$), 9.03 (1 H, t, $J$ = 5.8 Hz, NH)

T.L.C. (C) Rf = 0.05

**N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (208)**

N-(Phenylmethoxycarbonyl)sarcosine (8.92 g, 40 mmol) was stirred with DCC (8.24 g, 40 mmol) and 2,4,5-trichlorophenol (7.9 g, 40 mmol) in EtOAc (100 ml) at 0°C for 4h. The suspension was filtered and the solvent was evaporated from the filtrate. The residue was redissolved in EtOAc and filtered. The solvent was evaporated from the filtrate to give N-(phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (13.73g, 85%) as a pale buff wax.

$^1$H NMR ($CDCl_3$)

δ 3.12 (3 H, s, $CH_3$), 4.37 (2 H, s, Sar-$H_2$), 5.19 (2 H, s, Ar-$CH_2$), 7.03 (1 H, s, Ar'-6H), 7.38 (5 H, m, Ar), 7.55 (1 H, s, Ar'-3H)

δ 3.14 (3 H, s, $CH_3$), 4.28 (2 H, s, Sar-$H_2$), 5.18 (2 H, s, Ar-$CH_2$), 7.12 (1 H, s, Ar'-6H), 7.38 (5 H, m, Ar), 7.56 (1 H, s, Ar'-3H)

T.L.C. (C) Rf = 0.88

**N-(N-Phenylalanylleucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide hydrochloride (210)**

Hydrogen chloride was bubbled through a solution of N-(N-(N-(1,1-dimethylethoxy-carbonyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide (950 mg, 1.4 mmol) in $CH_2Cl_2$ (50 ml) for 30 min. The solvent and excess hydrogen chloride were evaporated to give N-(N-phenylalanylleucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (820 mg, 95%) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.85 (3 H, d, *J* = 6.6 Hz, Leu-$CH_3$), 0.88 (3 H, d, *J* = 6.6 Hz, Leu-$CH_3$), 1.51 (2 H, m, Leu β-$H_2$), 1.61 (1 H, m, Leu γ-H), 2.87 (3 H, s, Sar-$CH_3$), 2.85 (2 H, m, Phe β-$H_2$), 3.11 (4 H, m, $NCH_2CH_2N$), 3.74-3.89 (4 H, br, Gly-$H_2$ + Sar-$H_2$), 4.07 (1 H, m, Leu α-H), 4.34 (1 H, m, Phe α-H), 5.03 (2 H, s, Ar-$CH_2$), 7.27 (10 H, m, Ar), 7.96 (2 H, m, 2 × NH), 8.24 (3 H, m, $NH_3^+$), 8.82 (2 H, m, 2 × NH)

δ 0.85 (3 H, d, *J* = 6.6 Hz, Leu-$CH_3$), 0.88 (3 H, d, *J* = 6.6 Hz, Leu-$CH_3$), 1.51 (2 H, m, Leu β-$H_2$), 1.61 (1 H, m, Leu γ-H), 2.85 (3 H, s, Sar-$CH_3$), 2.88 (2 H, m, Phe β-$H_2$), 3.11 (4 H, m, $NCH_2CH_2N$), 3.74 (2 H, m, Gly-$H_2$), 3.89 (2 H, m, Sar-$H_2$), 4.07 (1 H, m, Leu α-H), 4.34 (1 H, m, Phe α-H), 5.07 (2 H, s, Ar-$CH_2$), 7.27 (10 H, m, Ar), 7.94 (1 H, m, NH), 8.03 (1 H, m, NH), 8.24 (3 H, m, $NH_3^+$), 8.82 (2 H, m, 2 × NH)

T.L.C. (C) Rf = 0.07

MS (FAB -) 617 (M + Cl)

Acc. Mass 583.1123 (M + H) ($C_{29}H_{20}N_6O_6$ requires 583.1133)

**N-(N-(N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (211)**

N-(N-phenylalanylleucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide (1.58 g, 2.55 mmol) was added to a stirred solution of N-(1,1-dimethylethoxy-carbonyl)glycine (904 mg, 2.55 mmol) and N,N-diisopropylethylamine (990 mg, 7.7 mmol) in $CH_2Cl_2$ (20 ml) and the mixture was stirred for 4 d. The solution was washed with cold 10% aq. $H_2SO_4$, with 10% aq. $Na_2CO_3$ and with brine. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 10 : 1) gave N-(N-(N-(N-(1,1-dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.14 g, 61%) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.83 (3 H, d, *J* = 6.2 Hz Leu-$H_3$), 0.88 (3 H, d, *J* = 6.2 Hz Leu-$H_3$), 1.36 (9 H, s, Bu-t), 1.49 (2 H, m, Leu β-$H_2$), 1.59 (1 H, m, Leu γ-H), 2.80 (1 H, dd, *J* = 13.5, 9 Hz, Phe β-$H_2$), 2.87 (3 H, s, Sar-$H_3$), 3.02 (1 H, dd, *J* = 13.5, 6 Hz, Phe β-$H_2$), 3.12 (4 H, br, $HCH_2CH_2N$), 3.38-3.62 (2 H, m, Gly-$H_2$), 3.66 (2 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.27 (1 H, m, Leu α-H), 4.55 (1 H, m, Phe α-H), 5.04 (2 H, s, Ar-$CH_2$), 6.94 (1 H, t, *J* = 4.5 Hz, NH), 7.37 (10 H, m, Ar), 7.80-8.12 (4 H, m, 4 × NH), 8.21 (1 H, d, *J* = 7.5 Hz)

δ 0.83 (3 H, d, *J* = 6.2 Hz Leu-$H_3$), 0.88 (3 H, d, *J* = 6.2 Hz Leu-$H_3$), 1.36 (9 H, s, Bu-t), 1.49 (2 H, m, Leu β-$H_2$), 1.59 (1 H, m, Leu γ-H), 2.80 (1 H, dd, *J* = 13.5, 9 Hz, β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.02 (1 H, dd, *J* = 13.5, 6 Hz, Phe β-$H_2$), 3.12 (4 H, br, $HCH_2CH_2N$), 3.38-3.62 (2 H, m, Gly-$H_2$), 3.66 (2 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.27 (1 H, m, Leu α-H), 4.55 (1 H, m, Phe α-H), 5.08 (2 H, s, Ar-$CH_2$), 6.94 (1 H, t, *J* = 4.5 Hz, NH), 7.37 (10 H, m, Ar), 7.80-8.12 (4 H, m, 4 × NH), 8.21 (1 H, d, *J* = 7.5 Hz)

T.L.C. (C) Rf = 0.39

MS (FAB+) 740 (M + H)

**N-(N-(N-Glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (212)**

Hydrogen chloride was bubbled through a solution of N-(N-(N-(N-(1,1-dimethylethoxy-carbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (1.29 g, 2.0 mmol) in $CH_2Cl_2$ (20 ml) for 30 min. MeOH (1 ml) was added and the solvents and excess hydrogen chloride were evaporated to give N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide hydrochloride (1.17 g, 73%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) (COSY 90)

δ 0.83 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 0.89 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 1.52 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.78 (1 H, dd, $J$ = 14, 10 Hz, Phe β-H), 2.86 (3 H, m, Sar-$CH_3$), 3.11 (5 H, m, $NCH_2CH_2N$ + Phe β-H), 3.61 (2 H, m, 2 x Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.32 (1 H, m, Leu α-H), 4.65 (1 H, m, Phe α-H), 5.07 (2 H, s, Ar-$CH_2$), 7.29 (11 H, m, Ar + Gly-NH), 7.95 (1 H, m, N<u>H</u>$CH_2CH_2NH$), 8.09 (4 H, br, $NH_3^+$ + $NHCH_2CH_2N$<u>H</u>), 8.49 (1 H, m, Leu-NH), 8.73 (1 H, m, Phe-NH)

δ 0.83 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 0.89 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 1.52 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.78 (1 H, dd, $J$ = 14, 10 Hz, Phe β-H), 2.86 (3 H, m, Sar-$CH_3$), 3.11 (5 H, m, $NCH_2CH_2N$ + Phe β-H), 3.61 (2 H, m, 2 x Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.32 (1 H, m, Leu α-H), 4.65 (1 H, m, Phe α-H), 5.04 (2 H, s, Ar-$CH_2$), 7.29 (11 H, m, Ar + Gly-NH), 7.95 (1 H, m, N<u>H</u>$CH_2CH_2NH$), 8.09 (4 H, br, $NH_3^+$ + $NHCH_2CH_2N$<u>H</u>), 8.49 (1 H, m, Leu-NH), 8.73 (1 H, m, Phe-NH)

T.L.C. (C) Rf = 0.10

MS (FAB+) 640 (M + H)

Acc. Mass 640.3480 (M + H) ($C_{32}H_{46}N_7O_7$ requires 640.3459)

**N-(N-(N-(N-(N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (213)**

N-(N-(N-Glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (1.11 g, 1.64 mmol) was added to N,N-diisopropyl-ethylamine (683 mg, 5.3 mmol) in $CH_2Cl_2$ (10 ml). N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester (950 mg, 1.77 mmol) and DMAP (10 mg) were added to this mixture and stirring continued for 3 d. The solution was washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$ The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 40:1 then $CHCl_3$ / MeOH 20:1 then $CHCl_3$ / MeOH 10:1) gave N-(N-(N-(N-(N$^{\alpha}$-(1,1-dimethyl-ethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.44 g, 78%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.25-1.65 (18 H, s + m, Bu-t + Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.75 (1 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.99 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.35 (2 H, s, Gly-$H_2$), 3.65 (2 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.84 (1 H, m, α-H), 4.25 (1 H, q, $J$ = 6 Hz, α-H), 4.55 (1 H, m, α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 6.87 (1 H, d, $J$ = 7 Hz, NH), 7.15-7.40 (10 H, m, Ar + Phe-Ar), 7.67 (1 H, t, $J$ = 6 Hz, NH), 7.81 (1 H, br, NH), 7.93-8.06 (3 H, m, 3 × NH), 8.20 (1 H, d, $J$ = 6 Hz, NH)

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.25-1.65 (18 H, s + m, Bu-t + Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.75 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 2.99 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.35 (2 H, s, Gly-$H_2$), 3.65 (2 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.84 (1 H, m, α-H), 4.25 (1 H, q, $J$ = 6 Hz, α-H), 4.55 (1 H, m, α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.07 (2 H, s, Ar-$CH_2$), 6.87 (1 H, d, $J$ = 7 Hz, NH), 7.15-7.40 (10 H, m, Ar + Phe-Ar), 7.67 (1 H, t, $J$ = 6 Hz, NH), 7.81 (1 H, br, NH), 7.93-8.06 (3 H, m, 3 × NH), 8.20 (1 H, d, $J$ = 6 Hz, NH)

T.L.C. (C) Rf = 0.48

MS (FAB+) 1045 (M + H).

**N-(N-(N-(N-(N$^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (214)**

Hydrogen chloride was bubbled through a solution of N-(N-(N-(N-(N$^{\alpha}$-(1,1-dimethylethoxycarbonyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.33 g, 1.27 mmol) in $CH_2Cl_2$ (10 ml) for 30 min. MeOH (1 ml) was added and the solvents and excess hydrogen chloride were evaporated to give N-(N-(N-(N-(N$^{\varepsilon}$-(2,2,2-trichloro-ethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide hydrochloride (1.14 g, 92%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.84 (3 H, d, *J* = 6.2 Hz, Leu-$CH_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$CH_3$), 1.28-1.67 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.80 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 3.04 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.59-3.91 (7 H, m, Sar-$H_2$ + 2 × Gly-$H_2$ + Lys α-H), 4.26 (1 H, m, Leu α-H), 4.56 (1 H, m, Phe α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 7.26 (11 H, m, Ar + NH), 7.69 (1 H, t, *J* = 5.3 Hz, NH), 7.93 (1 H, br, NH), 8.11 (2 H, m, 2 × NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.70 (1 H, t, *J* = 5.3 Hz, NH).

δ 0.84 (3 H, d, *J* = 6.2 Hz, Leu-$CH_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$CH_3$), 1.28-1.67 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.80 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.04 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.59-3.91 (7 H, m, Sar-$H_2$ + 2 × Gly-$H_2$ + Lys α-H), 4.26 (1 H, m, Leu α-H), 4.56 (1 H, m, Phe α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 7.26 (11 H, m, Ar + NH), 7.69 (1 H, t, *J* = 5.3 Hz, NH), 7.93 (1 H, br, NH), 8.11 (2 H, m, 2 × NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.70 (1 H, t, *J* = 5.3 Hz, NH).

T.L.C. (C) Rf = 0.12

MS (FAB+) 944 (M + H)

Acc. Mass 942.3453 (M + H) ($C_{41}H_{59}{}^{35}Cl_3N_9O_{10}$ requires 942.3450)

**N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (215)**

N-(N-(N-(N-(N$^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (980 mg, 1.0 mmol) was added to N,N-diisopropylethylamine (402 mg, 3.0 mmol) in $CH_2Cl_2$ (30 ml). N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (418 mg, 1 mmol) and DMAP (10 mg) were added to the mixture and the resulting solution was stirred for 20 h. The solution was washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CHCl_3$ / MeOH 20 : 1 then $CHCl_3$ / MeOH 10 : 1) gave N-(N-(N-(N-(N$^{\alpha}$-(N-(phenyl-methoxycarbonyl)sarcosyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (719 mg, 62%) as a friable foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.23-1.59 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.76 (1 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.86 (3 H, s, Sar-$H_3$), 2.99 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.64 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 3.90 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, Lys α-H + Leu α-H), 4.54 (1 H, m, Phe α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 5.07 (2 H, br, Ar-$CH_2$), 7.36 (15 H, m, Ar), 7.68 (1 H, m, NH), 7.82 (1 H, m, NH), 8.02 (2 H, m, 2 × NH), 8.18 (4 H, br, 4 × NH)

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.23-1.59 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.76 (1 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 2.99 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$),

3.64 (4 H, m, 2 × Gly-$H_2$), 3.81 (2 H, s, Sar-$H_2$), 3.90 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, Lys α-H + Leu α-H), 4.54 (1 H, m, Phe α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 5.07 (2 H, br, Ar-$CH_2$), 7.36 (15 H, m, Ar), 7.68 (1 H, m, NH), 7.82 (1 H, m, NH), 8.02 (2 H, m, 2 × NH), 8.18 (4 H, br, 4 × NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.85 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.89 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.29-1.69(9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.85 (1 H, m, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 3.01 (2 H, m, Lys ε-$H_2$), 3.07 (1 H, m, Phe β-$H_2$), 3.16 (4 H, br, $NCH_2CH_2N$), 3.75 (2 H, m, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 3.93 (2 H, s, Sar-$H_2$), 4.26 (2 H, m, Leu α-H, Lys α-H), 4.53 (1 H, m, Phe α-H), 5.05 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.34 (11 H, m, Ar + NH), 7.54 (1 H, br, NH), 7.68 (2 H, m, 2 × NH), 7.82 (1 H, m, NH), 7.84 (2 H, m, 2 × NH), 7.90 (1 H, m, NH)

T.L.C. (C) Rf = 0.35

MS (FAB+) 1149 (M + H)

Acc. Mass 1147.4211 (M + H) ($C_{52}H_{70}{}^{35}Cl_3N_{10}O_{13}$ requires 1147.4189)

**N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (216)**

N-(N-(N-Glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (4.47 g, 4.6 mmol) was added to N,N-diisopropyl-ethylamine (2.72 g, 21.0 mmol) in $CH_2Cl_2$ (50 ml). N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (7.48 g, 13.8 mmol) and DMAP (20 mg) were added to the mixture and the resulting solution was stirred for 2 d. The solution was washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20 : 1 then $CH_2Cl_2$ / MeOH 10 : 1) gave N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide(3.50 g, 65%) as a pale cream foam.

$^1$H NMR ($(CD_3)_2SO$) 20 °C (COSY 90)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 2.49 (2 H, m, Leu β-$H_2$), 1.61 (1 H, m, Leu γ-H), 2.77 (1 H, m, Phe β-$H_2$), 2.82 (3 H, s, Sar-$H_3$), 2.86 (3 H, s, Sar-$H_3$), 3.03 (1 H, m, Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.61-3.77 (4 H, m, 2 × Gly-$H_2$), 3.82 (2 H, s, Sar-$H_2$), 3.87 (2 H, s, Sar-$H_2$), 4.25 (1 H, m, Leu α-H), 4.53 (1 H, br, Phe α-H), 5.01 (2 H, s, Ar-$CH_2$), 5.06 (2 H, s, Ar-$CH_2$), 7.22 (5 H, m, Ar), 7.35 (5 H, m, Ar), 7.79 (1 H, m, NH), 8.06 (2 H, br, HNCH$_2$CH$_2$NH), 8.11 (1 H, m, Gly-NH), 8.16 (1 H, br, Gly-NH)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 2.49 (2 H, m, Leu β-$H_2$), 1.61 (1 H, m, Leu γ-H), 2.77 (1 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.88 (3 H, s, Sar-$H_3$), 3.03 (1 H, m, Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.61-3.77 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 3.88 (2 H, s, Sar-$H_2$), 4.25 (1 H, m, Leu α-H), 4.53 (1 H, br, Phe α-H), 5.02 (2 H, s, Ar-$CH_2$), 5.06 (2 H, s, Ar-$CH_2$), 7.22 (5H, m, Ar), 7.35 (5 H, m, Ar), 7.79 (1 H, m, NH), 8.06 (2 H, br, HNCH$_2$CH$_2$NH), 8.11 (1 H, m, Gly-NH), 8.16 (1 H, br, Gly-NH)

$^1$H NMR ($(CD_3)_2SO$) 80 °C

δ 0.84 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.51 (2 H, m, Leu β-$H_2$), 1.62 (1 H, m, Leu γ-H), 2.82 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 2.95 (3 H, s, Sar-$H_3$), 3.08 (1 H, m, Phe β-$H_2$), 3.15 (4 H, br, $NCH_2CH_2N$), 3.63 (3 H, Gly-$H_2$), 3.76 (1 H, m, Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 3.89 (2 H, s, Sar-$H_2$), 4.25 (1 H, m, Leu α-H), 4.55 (1 H, m, Phe α-H), 5.06 (2 H, s, Ar-$CH_2$), 7.22 (5 H, m, Ar), 7.33 (5 H, m, Ar), 7.54 (2 H, br, HNCH$_2$CH$_2$NH), 7.68 (2 H, br, 2 × Gly-NH), 7.88 (2 H, m, Phe-NH + Leu-NH)

T.L.C. (C) Rf = 0.32

Acc. Mass 845.4216 (M + H) ($C_{43}H_{57}N_8O_{10}$ requires 845.4198)

**$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine pentafluorophenyl ester (217)**

DCC (4.72 g, 22.9 mmol) and pentafluorophenol (4.21 g, 22.9 mmol) were added to a stirred solution of $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)-glutamine (8.57 g, 22.9 mmol) in EtOAc (150 ml). The resulting suspension was stirred

for 18 h and was then filtered. The filtrate was evaporated and the residue was re-dissolved in EtOAc and filtered. The solvent was evaporated to give $N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine pentafluorophenyl ester (12.37 g, quantitative) as a pale yellow wax.

$^1$H NMR ($CDCl_3$)

δ 1.23 (2 H, m, $NCH_2CH_2\underline{CH_2}CH_2CH_2$), 1.36 (11 H, m, Bu-t + $NCH_2\underline{CH_2}CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2\underline{CH_2}CH_2$), 1.63 (2 H, m, Glu β-$H_2$), 2.14 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2\underline{CH_2}$), 3.04 (2 H, q, $J$ = 7 Hz, $N\underline{CH_2}CH_2CH_2CH_2CH_2$), 3.60 (3 H, s, $OCH_3$), 3.88 (1 H, m, Glu α-H), 6.92 (1 H, d, $J$ = 7.6 Hz, Glu-NH), 7.23 (10 H, m, Ar), 7.75 (1 H, t, $J$ = 5.2 Hz, $N\underline{H}CH_2CH_2CH_2$).

$^{19}$F NMR ($CDCl_3$)

δ -152.6 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -158.7 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.5 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

T.L.C. (C) Rf = 0.56

**N-(N-(N-(N-($N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl) amide (218)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine pentafluorophenyl ester (12.36 g, 22.9 mmol) was added to a stirred solution of N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide hydrochloride (5.14 g, 7.6 mmol), N,N-diisopropylethylamine (2.17 g, 16.8 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (40 ml). After 18 h, dimethylformamide (20 ml) was added and stirring continued for 3 d. The solvent was evaporated and the residue was dissolved in EtOAc / MeOH (1:1). The solution was washed twice with cold 10% aq. $H_2SO_4$, and twice with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1 then $CH_2Cl_2$ / MeOH 15:1) gave N-(N-(N-(N-

solvent was evaporated and the residue was dissolved in EtOAc. The solution was filtered and the solvent was evaporated to give pentafluorophenyl 6-(1,1-dimethylethoxy-carbonylamino)hexanoate (21.91 g, 98%) as a cream wax.

$^1$H NMR ($CDCl_3$)

δ 1.44 (11 H, m, Bu-t + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.54 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.79 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.68 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.14 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 4.58 (1 H, br, NH)

$^{19}$F NMR ($CDCl_3$)

δ -153.3 (2 F, d, $J$ = 21.5 Hz, 2,6-Ar), -158.6 (1 F, t, $J$ = 18.1 Hz, 4-Ar), -162.8 (2 F, t, $J$ = 20 Hz, 3,5-Ar).

T.L.C. (C) Rf = 0.62

**$N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(phenylmethoxy-carbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide (245)**

Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (1.51 g, 4.79 mmol) was added to $N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (1.9 g, 3.19 mmol), N,N-dimethylethylamine (1.12 g, 12.76 mmol) and DMAP in $CH_2Cl_2$ (20 ml) and DMF (10 ml). The solution was stirred for 24 h and Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (1.51 g, 4.79 mmol) was added. The solution was stirred for 3 d. The solvents were evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1) yielded $N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonyl-amino)hexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (2.18 g, 85%) as a buff foam.

($N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)-phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl) amide (6.41 g, 85%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 1.23 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (11 H, m, Bu-t + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$ + Leu β-$H_2$), 1.57 (1 H, m, Leu γ-H), 1.63 (2 H, m, Glu β-$H_2$), 2.14 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 3.04 (2 H, q, $J$ = 7 Hz, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.12 (5 H, m, $NCH_2CH_2N$ + Phe β-$H_2$), 3.57 (3 H, s, $OCH_3$), 3.60-3.69 (4 H., m, 2 × Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 3.88 (1 H, m, Glu α-H), 4.25 (1 H, m, Leu α-H), 4.54 (1 H, br, Phe α-H), 5.03 (2 H, s, Ar-$CH_2$), 6.92 (1 H, d, $J$ = 7.6 Hz, Glu-NH), 7.23 (10 H, m, Ar), 7.75 (1 H, t, $J$ = 5.2 Hz, $N\underline{H}CH_2CH_2CH_2$), 7.81 (1 H, br, $NHCH_2CH_2NH$), 7.99 (3 H, br, 2 × Gly-NH + $NHCH_2CH_2NH$), 8.05 (1 H, d, $J$ = 8 Hz, Phe-NH), 8.18 (1 H, m, Leu-NH)

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$CH_3$), 1.23 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (11 H, m, Bu-t + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$ + Leu β-$H_2$), 1.57 (1 H, m, Leu γ-H), 1.63 (2 H, m, Glu β-$H_2$), 2.14 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.04 (2 H, q, $J$ = 7 Hz, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.12 (5 H, m, $NCH_2CH_2N$ + Phe β-$H_2$), 3.57 (3 H, s, $OCH_3$), 3.60-3.69 (4 H., m, 2 × Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 3.88 (1 H, m, Glu α-H), 4.25 (1 H, m, Leu α-H), 4.54 (1 H, br, Phe α-H), 5.07 (2 H, s, Ar-$CH_2$), 6.92 (1 H, d, $J$ = 7.6 Hz Glu-NH), 7.23 (10 H, m, Ar), 7.75 (1 H, t, $J$ = 5.2 Hz, $N\underline{H}CH_2CH_2CH_2$), 7.81 (1 H, br, $NHCH_2CH_2NH$), 7.99 (3 H, br, 2 × Gly-NH + $NHCH_2CH_2NH$), 8.05 (1 H, d, $J$ = 8 Hz, Phe-NH), 8.18 (1 H, m, Leu-NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.86 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 0.90 (3 H, d, $J$ = 6.1 Hz, Leu-$CH_3$), 1.29 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.39 (11 H, m, Bu-t + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.53 (4 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$ + Leu β-$H_2$), 1.63 (1 H, m, Leu γ-H), 1.75 (2 H, m, Glu β-$H_2$), 2.13 (2 H, m, Glu γ-$H_2$), 2.28 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.85 (1 H, m, Phe β-$H_2$), 2.90 (3 H, s, Sar-$H_3$), 3.05 (2 H, q, $J$ = 6.6 Hz, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.11 (5 H, m, $NCH_2CH_2N$ + Phe β-$H_2$), 3.59 (3 H, s, $OCH_3$), 3.68 (4 H., m, 2 × Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 3.89 (1 H, m, Glu α-H), 4.26 (1 H, m, Leu α-H), 4.55 (1 H, br, Phe α-H), 5.07 (2 H, s, Ar-$CH_2$), 6.57 (1 H, br, Glu-NH), 7.23 (10 H, m, Ar), 7.51 (1 H, m,

N<u>H</u>$CH_2CH_2CH_2$), 7.56 (1 H, br, $NHCH_2CH_2NH$), 7.69 (3 H, br, 2 × Gly-NH + $NHCH_2CH_2NH$), 7.78 (1 H, m, Phe-NH), 7.87 (1 H, m, Leu-NH)

T.L.C. (C) Rf = 0.33

**N-(N-(N-(N-(N$^{\delta}$-(6-Methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (219)**

Hydrogen chloride was bubbled through a solution of N-(N-(N-(N-(N$^{\alpha}$-(1,1-dimethylethoxycarbonyl)-N$^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.13 g, 3.15 mmol) in $CH_2Cl_2$ (20 ml) and MeOH (2 ml) for 30 min. The solvents and excess hydrogen chloride were evaporated to give N-(N-(N-(N-(N$^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.93 g, quantitative) as a beige foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.83 (3 H, d, *J* = 6.1 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.1 Hz, Leu-$H_3$), 1.23 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.35 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.51 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.60 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.68 (2 H, m, Glu β-$H_2$), 2.20 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, *J* = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.79 (1 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 3.09 (7 H, br, $NCH_2CH_2N$ + $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Phe β-$H_2$), 3.32 (1 H, m, Glu α-H), 3.57 (3 H, s, $OCH_3$), 3.65 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 4.64 (1 H, m, Leu α-H), 4.93 (1 H, m, Phe α-H), 5.03 (2 H, s, Ar-$CH_2$), 7.17 (1 H, m, NH), 7.27 (10 H, m, Ar), 7.91 (1 H, br, NH), 8.00 (1 H. m. NH), 8.17 (1 H, br, NH), 8.33 (5 H, br, $NH_3^+$ + 2 × NH), 8.72 (1 H, m, NH).

δ 0.83 (3 H, d, *J* = 6.1 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.1 Hz, Leu-$H_3$), 1.23 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.35 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.51 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.60 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.68 (2 H, m, Glu β-$H_2$), 2.20 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, *J* = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.79 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.09 (7 H, br, $NCH_2CH_2N$ + $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Phe β-$H_2$), 3.32 (1 H, m, Glu α-H), 3.57 (3 H, s, $OCH_3$), 3.65 (4 H, m, 2 × Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.64 (1 H, m, Leu α-H), 4.93 (1 H, m, Phe α-H), 5.07 (2 H, s, Ar-$CH_2$),

7.17 (1 H, m, NH), 7.27 (10 H, m, Ar), 7.91 (1 H, br, NH), 8.00 (1 H. m. NH), 8.17 (1 H, br, NH), 8.33 (5 H, br, $NH_3^+$ + 2 × NH), 8.72 (1 H, m, NH).

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.84 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.27 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.41 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.55 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.60 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.68 (2 H, m, Glu β-$H_2$), 2.26 (2 H, m, Glu γ-$H_2$), 2.48 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.88 (3 H, s, Sar-$H_3$ + Phe β-$H_2$), 3.11 (2H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.18 (5 H, br, $NCH_2CH_2N$ + Phe β-$H_2$), 3.36 (1 H, m, Glu α-H), 3.58 (3 H, s, $OCH_3$), 3.65 (4 H, m, 2 × Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.64 (1 H, m, Leu α-H), 4.93 (1 H, m, Phe α-H), 5.06 (2 H, s, Ar-$CH_2$), 7.17 (1 H, m, NH), 7.27 (10 H, m, Ar), 7.78 (3 H, br, 3 × NH), 8.10 (1 H. m, NH), 8.13 (1 H, m, NH), 8.30 (3 H, br, $NH_3^+$), 8.72 (1 H, m, NH).

T.L.C. (C) Rf = 0.10

**N-(N-(N-(N-(N$^{\delta}$-(6-Methoxy-6-oxohexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl-sarcosyl)amino)ethyl)amide (220)**

N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (7.23 g, 18.6 mmol) was added to a stirred solution of N-(N-(N-(N-(N$^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)-glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide hydrochloride (5.77 g, 6.2 mmol), N,N-diisopropylethylamine (3.20 g, 24.8 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (30 ml). After 18 h, dimethylformamide (20 ml) was added and stirring continued for 4d. The solvents were evaporated and the residue was dissolved in EtOAc / MeOH (1:1). The solution was washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1 then $CH_2Cl_2$ / MeOH 15:1 then $CH_2Cl_2$ / MeOH 10:1 ) gave N-(N-(N-(N-(N$^{\delta}$-(6-methoxy-6-oxohexyl)-N$^{\alpha}$-(N-(phenyl-methoxycarbonyl)sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonylsarcosyl)amino)ethyl)amide (3.19 g, 47%) as a pale yellow foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu $H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu $H_3$), 1.22 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.27 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.62 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.69 (2 H, m, Glu β-$H_2$), 2.08 (2 H, m, Glu γ-$H_2$), 2.27 (3 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.74 (1 H, m, Phe β-$H_2$), 2.84 (2 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 2.94 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.66 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 3.92 (2 H, s, Sar-$H_2$), 4.26 (2 H, m, Leu α-H + Glu α -H), 4.53 (1 H, m, Phe α-H), 5.01 (2 H, s, Ar-$CH_2$), 5.07 (2H, s, Ar-$CH_2$), 7.29 (15 H, m, Ar), 7.78 (1 H, m, $N\underline{H}CH_2CH_2CH_2$), 7.83 (1 H, m, $\underline{H}NCH_2CH_2NH$), 8.08 (2 H, m, $HNCH_2CH_2N\underline{H}$ + Gly-NH), 8.12 (1 H, m, Phe-NH), 8.32 (3 H, m, Leu-NH, Gly NH, Glu-NH)

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.22 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.27 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.62 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.69 (2 H, m, Glu β-$H_2$), 2.08 (2 H, m, Glu γ-$H_2$), 2.27 (3 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.74 (1 H, m, Phe β-$H_2$), 2.86 (2 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 2.94 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.66 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 3.92 (2 H, s, Sar-$H_2$), 4.26 (2 H, m, Leu α-H + Glu α -H), 4.53 (1 H, m, Phe α-H), 5.03 (2 H, s, Ar-$CH_2$), 5.04 (2H, s, Ar-$CH_2$), 7.29 (15 H, m, Ar), 7.78 (1 H, m, $N\underline{H}CH_2CH_2CH_2$), 7.83 (1 H, m, $\underline{H}NCH_2CH_2NH$), 8.08 (2 H, m, $HNCH_2CH_2N\underline{H}$ + Gly-NH), 8.12 (1 H, m, Phe-NH), 8.32 (3 H, m, Leu-NH, Gly NH, Glu-NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.84 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.26 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.39 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.57 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.62 (3 H, m, Leu γ-H + Leu β-$H_2$), 1.75 (2 H, m, Glu β-$H_2$), 2.12 (2 H, t, $J$ = 7.6 Hz, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.85 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.09 (7 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + $NCH_2CH_2N$), 3.58 (3 H, s, $OCH_3$), 3.66 (4 H, m, 2 × Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 3.94 (2 H, br, Sar-$H_2$), 4.24 (2 H, m, Leu α-H + Glu α-H), 4.54 (1 H, m, Phe α-H), 5.05 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.28 (15 H, m, Ar), 7.48 (1 H, m, NH), 7.58 (1 H, br, NH), 7.71 (2 H, m, 2 × NH), 7.84 (1 H, m, NH), 7.92 (3 H, m, 3 × NH), 8.01 (1 H, m, NH)

T.L.C. (C) Rf = 0.31

MS (FAB+) 1101 (M + H)

**$N^{\alpha}$-(N-(1,1-Dimethylethoxycarbonyl)glycyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (221)**

$N^{\delta}$-(6-Methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide hydrochloride (5.92 g, 10.6 mmol) was added to a stirred solution of N-(1,1-dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (3.76 g, 10.6 mmol) in $CH_2Cl_2$ (200 ml) containing N,N-diisopropylethylamine (4.11 g, 31.9 mmol) and DMAP (10 mg). The solution was stirred for 10 d. The solvent was evaporated and the residue was dissolved in EtOAc and then washed twice with cold 10% aq. $H_2SO_4$ and twice with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated to give a pink oil. Trituration with $Et_2O$ yielded $N^{\alpha}$-(N-(1,1-dimethylethoxycarbonyl)glycyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide (5.32 g, 74%) as a buff solid. Mp 100-102°C

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 1.22 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.37 (11 H, m, Bu-t + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.49 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.72 (2 H, m, Glu β-$H_2$), 2.05 (2 H, m, Glu γ-$H_2$), 2.24 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.86 (3 H, s, Sar-$H_3$), 3.10 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.37 (4 H, br, $NCH_2CH_2N$), 3.44 (2 H, m, Gly-$H_2$), 3.56 (3 H, s, $OCH_3$), 3.82 (2 H, s, Sar-$H_2$), 4.18 (1 H, m, Glu α-H), 5.04 (2 H, s, Ar-$CH_2$), 7.01 (1 H, m, NH), 7.36 (5 H, m, Ar), 7.76 (1 H, br, NH), 7.98 (3 H, br, 3 × NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 1.25 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.38 (11 H, m, Bu-t + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.51 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.76 (2 H, m, Glu β-$H_2$), 1.95 (2 H, m, Glu γ-$H_2$), 2.08 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.88 (3 H, s, Sar-$H_3$), 3.12 (2 H, q, $J$ = 6.5 Hz, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.25 (4 H, m, $NCH_2CH_2N$), 3.58 (5 H, m, Gly-$H_2$ + $OCH_3$), 3.83 (2 H, s, Sar-$H_2$), 4.20 (1 H, m, Glu α-H), 5.06 (2 H, s, Ar-$CH_2$), 6.62 (1 H, br, NH), 7.33 (6 H, m, Ar + NH), 7.46 (1 H, m, NH), 7.67 (2 H, br, 2 × NH).

T.L.C. (C) Rf = 0.56

MS (FAB+) 679 (M + H)

**$N^{\alpha}$-Glycyl-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide hydrochloride (222)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(1,1-dimethylethoxy-carbonyl)glycyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (3.86 g, 5.7 mmol) in tetrahydrofuran (100 ml) and MeOH (5 ml) for 30 min. The solvents and excess reagent were evaporated to give $N^{\alpha}$-glycyl-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (3.49 g, quantitative) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 1.22 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.47 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.72 (2 H, m, Glu β-$H_2$), 1.92 (2 H, m, Glu γ-$H_2$), 2.09 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.87 (3 H, s, Sar-$H_3$), 3.02 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.16 (4 H, br, $NCH_2CH_2N$), 3.6 (2 H, m, Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 4.22 (1 H, m, Glu α-H), 5.03 (2 H, s, Ar-$CH_2$), 7.26 (5 H, m, Ar), 7.89 (1 H, m, NH), 8.19 (3 H, br, $NH_3^+$), 8.67 (1 H, m, NH), 9.02 (1 H, br, NH)

δ 1.22 (2 H, m, $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (2 H, m, $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.47 (2 H, m, $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.72 (2 H, m, Glu β-$H_2$), 1.92 (2 H, m, Glu γ-$H_2$), 2.09 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.88 (3 H, s, Sar-$H_3$), 3.02 (2 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.16 (4 H, br, $NCH_2CH_2N$), 3.6 (2 H, m, Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 4.22 (1 H, m, Glu α-H), 5.07 (2 H, s, Ar-$CH_2$), 7.26 (5 H, m, Ar), 7.89 (1 H, m, NH), 8.19 (3 H, br, $NH_3^+$), 8.67 (1 H, m, NH), 9.02 (1 H, br, NH)

T.L.C. (C) Rf = 0.08

**$N^{\alpha}$-(N-(1,1-Dimethylethoxycarbonyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (224)**

$N^{\varepsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (6.3 g, 10.4 mmol) was added to N-(1,1-dimethyl-ethoxycarbonyl)glycine pentafluorophenyl ester (3.56 g, 10.4 mmol) and N,N-diiso-propylethylamine (4.05 g, 31.3 mmol) in $CH_2Cl_2$ (50 ml) and the solution was stirred for

3d. The solution was washed twice with cold 10% aq. $H_2SO_4$ and once with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated to give $N^{\alpha}$-(N-(1,1-dimethylethoxycarbonyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (6.89 g, 91%) as a pale brown foam

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 1.24 (2 H, m, Lys β,γ,δ-$H_2$), 1.39 (11 H, m, Bu-t + Lys β,γ,δ-$H_2$), 1.62 (2 H, m, Lys β,γ,δ-$H_2$), 2.87 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.58 (2 H, br, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.18 (1 H, m, Lys α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.05 (2 H, s, Ar-$CH_2$), 6.98 (1 H, m, NH), 7.37 (5 H, m, Ar), 7.68 (1 H, m, NH), 7.87 (1 H, d, $J$ = 8.6 Hz, Lys NH), 7.99 (2 H, br, 2 × NH)

δ 1.24 (2 H, m, Lys β,γ,δ-$H_2$), 1.39 (11 H, m, Bu-t + Lys β,γ,δ-$H_2$), 1.62 (2 H, m, Lys β,γ,δ-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.58 (2 H, br, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.18 (1 H, m, Lys α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 6.98 (1 H, m, NH), 7.37 (5 H, m, Ar), 7.68 (1 H, m, NH), 7.87 (1 H, d, $J$ = 8.6 Hz, Lys NH), 7.99 (2 H, br, 2 × NH)

T.L.C. (C) Rf = 0.42

MS (FAB-) 625 (M - Boc)

**$N^{\alpha}$-Glycyl-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (225)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(1,1-dimethylethoxycarbonyl)glycyl-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (5.87 g, 8.1 mmol) in $CH_2Cl_2$ (50 ml) for 30 min. The solvent and excess hydrogen chloride were evaporated to give $N^{\alpha}$-glycyl-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide (5.27 g, 98%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 1.26 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.63 (2 H, m, Lys β,γ,δ-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.99 (2 H, m, Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.60 (2 H, m, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.21 (1 H, m, Lys α-$H_2$), 4.78 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 7.69 (1 H, m, NH), 8.11 (3 H, br, $NH_3^+$), 8.18 (2 H, br, 2 × NH), 8.61 (1 H, d, $J$ = 7.6 Hz, Lys NH)

δ 1.26 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.63 (2 H, m, Lys β,γ,δ-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 2.99 (2 H, m, Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.60 (2 H, m, Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.21 (1 H, m, Lys α-$H_2$), 4.78 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 7.35 (5 H, m, Ar), 7.69 (1 H, m, NH), 8.11 (3 H, br, $NH_3^+$), 8.18 (2 H, br, NH), 8.61 (1 H, d, $J$ = 7.6 Hz, Lys NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 1.31 (2 H, m, Lys β,γ,δ-$H_2$), 1.45 (2 H, m, Lys β,γ,δ-$H_2$), 1.60 (2 H, m, Lys β,γ,δ-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.04 (2 H, m, Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.59 (2 H, m, Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.21 (1 H, m, Lys α-$H_2$), 4.75 (2 H, s, $CH_2CCl_3$), 5.07 (2 H, s, Ar-$CH_2$), 7.33 (5 H, br, Ar), 7.83 (1 H, m, NH), 7.92 (1 H, m, NH), 8.09 (3 H, br, $NH_3^+$), 8.43 (1 H, d, $J$ = 7.6 Hz, Lys NH).

T.L.C. (C) Rf = 0.12

**N-(1,1-Dimethylethoxycarbonyl)leucine pentafluorophenyl ester (226)**

N-(1,1-Dimethylethoxycarbonyl)leucine (63.56 g, 275 mmol) in EtOAc (200 ml) was cooled to 0°C. DCC (56.74 g, 275 mmol) and pentafluorophenol (50.62 g, 275 mmol) were added and the suspension was stirred for 24 h. The suspension was filtered and the solvent evaporated to give N-(1,1-dimethylethoxycarbonyl)leucine pentafluorophenyl ester (109 g, quantitative) as a cream wax.

$^1$H NMR ($CDCl_3$)

δ 0.89 (6 H, d, $J$ = 6.7 Hz, 2 × Leu-$H_3$), 1.47 (9 H, s, Bu-t), 1.72 (1 H, m, Leu β-$H_2$), 1.78 (2 H, m, Leu β-$H_2$ + Leu γ-H), 4.62 (1 H, m, Leu α-H), 4.95 (1 H, m, NH)

$^{19}$F NMR ($CDCl_3$)

δ -152.8 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -158.1 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.8 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

δ -153.2 (2 F, d, $J$ = 20 Hz, 2,6-Ar), -157.9 (1 F, t, $J$ = 20 Hz, 4-Ar), -162.6 (2 F, t, $J$ = 20 Hz, 3,5-Ar)

T.L.C. (C) Rf = 0.91

**$N^{\alpha}$-(N-(N-(1,1-Dimethylethoxycarbonyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (227)**

$N^{\alpha}$-Glycyl-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (5.14 g, 7.8 mmol) was added to a stirred solution of N-(1,1-dimethylethoxycarbonyl)leucine pentafluorophenyl ester (3.89 g, 7.8 mmol), N,N-diisopropylethylamine (3.01 g, 23.3 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (50 ml) The mixture was stirred for 4 d, then washed twice with cold 10% aq. $H_2SO_4$, once with 10% aq. $Na_2CO_3$ and once with brine. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 30:1 increasing to $CH_2Cl_2$ / MeOH 1:1) gave $N^{\alpha}$-(N-(N-(1,1-dimethylethoxycarbonyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (5.53 g, 85%) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.22-1.63 (18 H, m, Bu-t + Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 3.04 (2 H, q, $J$ = 6.9 Hz, Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.72 (2 H, d, $J$ = 5.1 Hz, Gly-$H_2$), 3.82 (2 H, s, Sar-$H_2$), 3.94 (1 H, m, Leu α-H), 4.14 (1 H, m, Lys α-H), 4.77 (2 H, s,

$CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 6.98 (1 H, d, $J$ = 8.1 Hz, NH), 7.36 (5 H, m, Ar), 7.64 (1 H, t, $J$ = 6.9 Hz, NH), 7.87 (1 H, d, $J$ = 8.6 Hz, NH), 7.95 (3 H, br, 3 × NH)

δ 0.83 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.22-1.63 (18 H, m, Bu-t + Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.04 (2 H, q, $J$ = 6.9 Hz, Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.72 (2 H, d, $J$ = 5.1 Hz, Gly-$H_2$), 3.82 (2 H, s, Sar-$H_2$), 3.94 (1 H, m, Leu α-H), 4.14 (1 H, m, Lys α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 6.98 (1 H, d, $J$ = 8.1 Hz, NH), 7.36 (5 H, m, Ar), 7.64 (1 H, t, $J$ = 6.9 Hz, NH), 7.87 (1 H, d, $J$ = 8.6 Hz, NH), 7.95 (3 H, br, 3 × NH)

T.L.C. (C) Rf = 0.41

MS (FAB+) 840 (M + H)

**$N^{\alpha}$-(N-Leucylglycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (228)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(N-(1,1-dimethylethoxycarbonyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (5.16 g, 6.15 mmol) in $CH_2Cl_2$ (50 ml) for 30 min. MeOH (2 ml) was added and the solvents and excess hydrogen chloride were evaporated to give $N^{\alpha}$-(N-(leucylglycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (4.76 g, quantitative) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.89 (6 H, m, 2 × Leu-$H_3$), 1.23 (2 H, br, Leu β-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.61 (5 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H), 2.86 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$CH_2$), 3.13 (4 H, br, $NCH_2CH_2N$), 3.84 (5 H, br, Gly-$H_2$ + Sar-$H_2$ + Leu α-H), 4.17 (1 H, m, Lys α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$H_2$), 7.37 (5 H, m, Ar), 7.67 (1 H, br, NH), 8.06 (1 H, br, NH), 8.13 (1 H, d, $J$ = 7.6 Hz, NH), 8.25 (3 H, br, $NH_3^+$), 8.81 (1 H, m, NH)

δ 0.89 (6 H, m, 2 × Leu-$H_3$), 1.23 (2 H, br, Leu β-$H_2$), 1.41 (2 H, m, Lys β,γ,δ-$H_2$), 1.61 (5 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H), 2.89 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$CH_2$), 3.13

(4 H, br, $NCH_2CH_2N$), 3.84 (5 H, br, Gly-$H_2$ + Sar-$H_2$ + Leu α-H), 4.17 (1 H, m, Lys α-H), 4.78 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$H_2$), 7.37 (5 H, m, Ar), 7.67 (1 H, br, NH), 8.06 (1 H, br, NH), 8.13 (1 H, d, $J$ = 7.6 Hz, NH), 8.25 (3 H, br, $NH_3^+$), 8.81 (1 H, m, NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.91 (6 H, m, 2 × Leu-$H_3$), 1.25 (2 H, br, Leu β-$H_2$), 1.43 (2 H, m, Lys β,γ,δ-$H_2$), 1.64 (5 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H), 2.89 (3 H, s, Sar-$H_3$), 3.00 (2 H, m, Lys ε-$CH_2$), 3.13 (4 H, br, $NCH_2CH_2N$), 3.85 (5 H, br, Gly-$H_2$ + Sar-$H_2$ + Leu α-H), 4.19 (1 H, m, Lys α-H), 4.75 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$H_2$), 7.33 (5 H, br, Ar), 7.81 (1 H, br, NH), 7.89 (1 H, d, $J$ = 8.2 Hz, NH), 8.22 (4 H, br, $NH_3^+$ + NH), 8.66 (1 H, t, $J$ = 5.2 Hz, NH)

T.L.C. (C) Rf = 0.14

MS (FAB+) 740 (M + H)

**$N^{\alpha}$-(N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (229)**

$N^{\alpha}$-(N-Leucylglycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (4.68 g, 6.0 mmol) was added to a stirred solution of N-(1,1-dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (2.60 g, 6.0 mmol), N,N-diisopropylethylamine (2.35 g, 18.1 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (50 ml). The mixture was stirred for 24 h, then washed twice with cold 10% aq. $H_2SO_4$, once with 10% aq. $Na_2CO_3$ and once with brine. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 30:1 then $CH_2Cl_2$ / MeOH 20:1) gave $N^{\alpha}$-(N-(N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (4.55 g, 77%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.83 (3 H, d, *J* = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.4 Hz, Leu-$H_3$), 1.23 (1 H, br, Leu β-$H_2$), 1.29 (13 H, Bu-t + Lys β-$H_2$ + Lys γ-$H_2$), 1.38 (2 H, m, Lys δ-$H_2$), 1.47 (1 H, m, Leu β-$H_2$), 1.64 (1 H, m, Leu γ-H), 2.73 (1 H, m, Phe β-$H_2$), 2.86 (3 H, Sar-$H_3$), 2.98 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.72 (2 H, d, *J* = 5.7 Hz, Gly-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.14 (2 H, m, Phe α-H + Lys α-H), 4.33 (1 H, m, Leu α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 6.94 (1 H, d, *J* = 8.3 Hz, Phe-NH), 7.27 (10 H, m, Ar), 7.66 (1 H, t, *J* = 5.4 Hz, Lys ε-NH)., 7.88 (1 H, d, *J* = 7.8 Hz, Lys α-NH), 7.96 (3 H, m, Leu-NH + $\underline{H}NCH_2CH_2N\underline{H}$), 8.07 (1 H, br, Gly-NH)

δ 0.83 (3 H, d, *J* = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.4 Hz, Leu-$H_3$), 1.23 (1 H, br, Leu β-$H_2$), 1.29 (13 H, Bu-t + Lys β-$H_2$ + Lys γ-$H_2$), 1.38 (2 H, m, Lys δ-$H_2$), 1.47 (1 H, m, Leu β-$H_2$), 1.64 (1 H, m, Leu γ-H), 2.73 (1 H, m, Phe β-$H_2$), 2.89 (3 H, Sar-$H_3$), 2.98 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.72 (2 H, d, *J* = 5.7 Hz, Gly-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.14 (2 H, m, Phe α-H + Lys α-H), 4.33 (1 H, m, Leu α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 6.94 (1 H, d, *J* = 8.3 Hz, Phe-NH), 7.27 (10 H, m, Ar), 7.66 (1 H, t, *J* = 5.4 Hz, Lys ε-NH)., 7.88 (1 H, d, *J* = 7.8 Hz, Lys α-NH), 7.96 (3 H, m, Leu-NH + $\underline{H}NCH_2CH_2N\underline{H}$), 8.07 (1 H, br, Gly-NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.85 (3 H, d, *J* = 6.4 Hz, 1.30 (14 H, Bu-t + Lys β-$H_2$ + Lys γ-$H_2$ + Leu β-$H_2$), 1.44 (2 H, m, Lys δ-$H_2$), 1.52 (1 H, m, Leu β-$H_2$), 1.65 (1 H, m, Leu γ-H), 2.78 (1 H, m, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 3.01 (3 H, Phe β-$H_2$ + Lys ε-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.72 (2 H, d, *J* = 5.8 Hz, Gly-$H_2$), 3.82 (2 H, s, Sar-$H_2$), 4.19 (2 H, Phe α-H + Lys α-H), 4.31 (1 H, m, Leu α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.28 (10 H, m, Ar), 7.58 (1 H, d, *J* = 7.9 Hz, NH), 7.65 (4 H, br, 4 × NH), 7.73 (1 H, d, *J* = 7.9 Hz, NH), 7.82 (1 H, t, *J* = 4.1 Hz, NH)

T.L.C. (C) Rf = 0.54

MS (FAB+) 987 (M + H)

**$N^{\alpha}$-(N-(N-Phenylalanylleucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (230)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(N-(N-(1,1-dimethylethoxy-carbonyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-

(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (4.24 g, 4.3 mmol) in $CH_2Cl_2$ (50 ml) for 30 min. MeOH (2 ml) was added and the solvents and excess hydrogen chloride were evaporated to give $N^{\alpha}$-(N-(N-phenylalanylleucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)-amide (3.96 g, quantitative) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.85 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.23-1.64 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.96 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.75 (2 H, m, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.13 (2 H, m, Phe α-H + Lys α-H), 4.22 (1 H, m, Leu α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$H_2$), 7.28 (10 H, m, Ar), 7.68 (1 H, t, $J$ = 5.2 Hz, NH), 7.97 (1 H, m, NH), 8.07 (2 H, br, NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.86 (1 H, d, $J$ = 7.9 Hz, NH)

δ 0.85 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.23-1.64 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 2.96 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.75 (2 H, m, Gly-$H_2$), 3.85 (2 H, s, Sar-$H_2$), 4.13 (2 H, m, Phe α-H + Lys α-H), 4.22 (1 H, m, Leu α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$H_2$), 7.28 (10 H, m, Ar), 7.68 (1 H, t, $J$ = 5.2 Hz, NH), 7.97 (1 H, m, NH), 8.07 (2 H, br, NH), 8.31 (4 H, br, $NH_3^+$ + NH), 8.86 (1 H, d, $J$ = 7.9 Hz, NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.85 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 1.24-1.73 (9 H, m, Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 3.03 (4 H, m , Phe β-$H_2$ + Lys ε-$H_2$), 3.13 (4 H, br, $NCH_2CH_2N$), 3.74 (2 H, m, Gly-$H_2$), 3.84 (2 H, s, Sar-$H_2$), 4.15 (2 H, m, Phe α-H + Lys α-H), 4.26 (1 H, m, Leu α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.28 (10 H, m, Ar), 7.68 (1 H, d, $J$ = 7.9 Hz, NH), 7.82 (1 H, br, NH), 7.99 (1 H, t, $J$ = 5.2 Hz, NH), 8.28 (4 H, br, $NH_3^+$ + NH), 8.71 (1 H, d, $J$ = 7.9 Hz, NH)

T.L.C. (C) Rf = 0.09

**$N^{\alpha}$-(N-(N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (231)**

$N^{\alpha}$-(N-(N-Phenylalanylleucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (3.75 g, 4.1 mmol) was added to a stirred solution of N-(1,1-dimethylethoxycarbonyl)glycine pentafluoro-phenyl ester (1.44 g, 4.1 mmol), N,N-diisopropylethylamine (1.58 g, 12.2 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (30 ml). The mixture was stirred for 12 d, then washed once with water, twice with cold 10% aq. $H_2SO_4$, once with 10% aq. $Na_2CO_3$ and once with brine. The solution was dried and the solvent was evaporated to give $N^{\alpha}$-(N-(N-(N-(N-(1,1-dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloro-ethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.94 g, 93%) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.23-1.41 (13 H, m, Bu-t + Lys β,γ-$H_2$), 1.47 (4 H, m, Lys δ-$H_2$ + Leu β-$H_2$), 1.59 (1 H, m, Leu γ-H), 2.78 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 3.01 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.5 (2 H, m, Gly-$H_2$), 3.73 (2 H, d, $J$ = 5.4 Hz, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.14 (1 H, m, Lys α-H), 4.28 (1 H, m, Leu α-H), 4.54 (1 H, m, Phe α-H), 4.77(2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 6.93 (1 H, t, $J$ = 5.9 Hz, Gly-NH), 7.27 (10 H, m, Ar), 7.66 (1 H, t, $J$ = 5.4 Hz, Lys ε-NH), 7.9 (4 H, br, Phe-NH + Lys-NH + $\underline{H}NCH_2CH_2N\underline{H}$), 8.06 (1 H, br, Gly-NH), 8.16 (1 H, d, $J$ = 7.3 Hz, Leu-NH)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.23-1.41 (13 H, m, Bu-t + Lys β,γ-$H_2$), 1.47 (4 H, m, Lys δ-$H_2$ + Leu β-$H_2$), 1.59 (1 H, m, Leu γ-H), 2.78 (1 H, m, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 3.01 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.5 (2 H, m, Gly-$H_2$), 3.73 (2 H, d, $J$ = 5.4 Hz, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.14 (1 H, m, Lys α-H), 4.28 (1 H, m, Leu α-H), 4.54 (1 H, m, Phe α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 6.93 (1 H, t, $J$ = 5.9 Hz, Gly-NH), 7.27 (10 H, m, Ar), 7.66 (1 H, t, $J$ = 5.4 Hz, Lys ε-NH), 7.9 (4 H, br, Phe-NH + Lys-NH + $\underline{H}NCH_2CH_2N\underline{H}$), 8.06 (1 H, br, Gly-NH), 8.16 (1 H, d, $J$ = 7.3 Hz, Leu-NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.84 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.38 (18 H, m, Bu-t + Leu γ-H + Leu β-$H_2$ + Lys β,γ,δ-$H_2$), 2.84 (1 H, m, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 3.02 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.51 (2 H, m, Gly-$H_2$), 3.72 (2 H, d, $J$ = 5.8 Hz, Gly-$H_2$), 3.83 (2 H, s, Sar-$H_2$), 4.16 (1 H, m, Lys α-H), 4.26 (1 H, m, Leu α-H), 4.55 (1 H, m, Phe α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.25 (10 H, m, Ar), 7.58 (1 H, d, $J$ = 7.9 Hz, NH), 7.65 (5 H, br, 5 × NH), 7.73 (1 H, t, $J$ = 5.5 Hz, NH), 7.85 (1 H, d, $J$ = 7.9 Hz, NH)

T.L.C. (C) Rf = 0.40

**$N^{\alpha}$-(N-(N-(N-Glycylphenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (232)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(N-(N-(N-(1,1-dimethyl-ethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.74 g, 1.67 mmol) in $CH_2Cl_2$ (30 ml) for 1 h. MeOH (1 ml) was added and the solvents and excess hydrogen chloride were evaporated to give $N^{\alpha}$-(N-(N-(N-glycylphenylalanyl)leucyl)glycyl-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide hydrochloride (1.64 g, quantitative) as a colourless foam.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.83 (3 H, d, $J$ = 6.0 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.0 Hz, Leu-$H_3$), 1.23 (2 H, m, Lys β,γ,δ–$H_2$), 1.39 (2 H, m, Lys β,γ,δ–$H_2$), 1.50-1.75 (5 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.77 (1 H, dd, $J$ = 13.5, 11.0 Hz, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.97 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.40-3.65 (2 H, m, Gly-$H_2$), 3.74 (2 H, br, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.29 (1 H, m, α-H), 4.62 (1 H, m, α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 7.18-7.40 (10 H, m, Ar + Phe-Ar), 7.68 (1 H, t, $J$ = 6 Hz, NH), 7.95 (1 H, d, $J$ = 8 Hz, NH), 8.00-8.15 (5 H, br, $NH_3^+$ + 2 × NH), 8.44 (1 H, d, $J$ = 7.9 Hz, NH), 8.70 (1 H, d, $J$ = 7.9 Hz, NH)

δ 0.83 (3 H, d, $J$ = 6.0 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.0 Hz, Leu-$H_3$), 1.23 (2 H, m, Lys β,γ,δ–$H_2$), 1.39 (2 H, m, Lys β,γ,δ–$H_2$), 1.50-1.75 (5 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.77 (1 H, dd, $J$ = 13.5, 11.0 Hz, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 2.97 (3 H, m, Phe β-$H_2$ + Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.40-3.65 (2 H, m, Gly-$H_2$), 3.74 (2 H, br, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.29 (1 H, m, α-H), 4.62 (1 H, m, α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.07 (2 H, s, Ar-$CH_2$), 7.18-7.40 (10 H, m, Ar + Phe-Ar), 7.68 (1 H, t, $J$ = 6 Hz, NH), 7.95 (1 H, d, $J$ = 8 Hz, NH), 8.00-8.15 (5 H, br, $NH_3^+$ + 2 × NH), 8.44 (1 H, d, $J$ = 7.9 Hz, NH), 8.70 (1 H, d, $J$ = 7.9 Hz, NH)

T.L.C. (C) Rf = 0.10

**$N^{\alpha}$-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (233)**

$N^{\alpha}$-(N-(N-(N-Glycylphenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (6.77 g, 6.9 mmol) was added to N,N-diisopropylethylamine (2.94 g, 22.7 mmol) in $CH_2Cl_2$ (50 ml). N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (8.07 g, 20.7 mmol) and DMAP (10 mg) were added to the mixture and the resulting solution was stirred for 2 d. The solution was washed twice with cold 10% aq. $H_2SO_4$, twice with 10% aq. $Na_2CO_3$ and once with brine. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1) gave $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxy-carbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (6.40 g, 81%) as a pale yellow foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.85 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.90 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.17 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (4 H, m, Lys β,γ,δ-$H_2$), 1.55 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.6-2.75 (1 H, m, Phe β-$H_2$), 2.77 (3 H, s, Sar-$H_3$), 2.81 (3 H, s, Sar-$H_3$), 2.93 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.08 (4 H, br, $NCH_2CH_2N$), 3.62 (4 H, m, 2 × Gly-$H_2$), 3.76 (2 H, br, Sar-$H_2$), 3.81 (2 H, br, Sar-$H_2$), 4.04 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.50 (1 H, m, α-H), 4.70 (2 H, s, $CH_2CCl_3$), 4.95 (2 H, s, Ar-$CH_2$), 5.00 (2 H, br, Ar-$CH_2$), 7.24

(15 H, m, 2 × Ar + Phe-Ar), 7.61 (1 H, m, NH), 7.92 (5 H, m, 5 × NH), 8.08 (3 H, m, 3 × NH)

δ 0.85 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.90 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.17 (2 H, m, Lys β,γ,δ-$H_2$), 1.41 (4 H, m, Lys β,γ,δ-$H_2$), 1.55 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.6-2.75 (1 H, m, Phe β-$H_2$), 2.79 (3 H, s, Sar-$H_3$), 2.82 (3 H, s, Sar-$H_3$), 2.93 (3 H, m, Lys ε-$H_2$ + Phe β-$H_2$), 3.08 (4 H, br, $NCH_2CH_2N$), 3.62 (4 H, m, 2 × Gly-$H_2$), 3.76 (2 H, br, Sar-$H_2$), 3.81 (2 H, br, Sar-$H_2$), 4.04 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.50 (1 H, m, α-H), 4.70 (2 H, s, $CH_2CCl_3$), 4.97 (2 H, s, Ar-$CH_2$), 5.00 (2 H, br, Ar-$CH_2$), 7.24 (15 H, m, 2 × Ar + Phe-Ar), 7.61 (1 H, m, NH), 7.92 (5 H, m, 5 × NH), 8.08 (3 H, m, 3 × NH).

T.L.C. (C) Rf = 0.42

Acc. Mass 1146.4133 (M + H) ($C_{52}H_{69}{}^{35}Cl_3N_{10}O_{13}$ requires 1146.4111)

**$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (234)**

$N^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutamine pentafluorophenyl ester (8.67 g, 16.05 mmol) was added to a stirred solution of $N^{\alpha}$-(N-(N-(N-glycylphenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (5.25 g, 5.35 mmol), N,N-dimethylethylamine (1.87 g, 21.4 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (40 ml) and dimethylformamide (10 ml). The solution was stirred for 4 d and then the solvent was evaporated. The residue was dissolved in EtOAc / MeOH (1:1), then washed with cold 10% aq. $H_2SO_4$, and with 10% aq. $Na_2CO_3$ . The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1 then $CH_2Cl_2$ / MeOH 15:1 then $CH_2Cl_2$ / MeOH 10:1 ) gave $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (5.11 g, 73%) as a pale buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.25 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.37 (13 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.50 (6 H, m, Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.62 (2 H, m, Leu γ-H + Glu β-H), 1.83 (1 H , m, Glu β-H), 2.07 (2 H, t, $J$ = 7.8 Hz, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76 (1 H, dd, $J$ = 15, 9 Hz, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.99 (5 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.64 (2 H, m, Gly-$H_2$), 3.72 (2 H, d, $J$ = 5.4 Hz, Gly-$H_2$) 3.83 (2 H, s, Sar-$H_2$), 3.88 (1 H, m, Leu α-H or Lys α-H), 4.16 (1 H, m. Leu α-H or Lys α-H), 4.23 (1 H, m, Glu α-H), 4.54 (1 H, m, Phe α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$CH_2$), 6.92 (1 H, d, $J$ = 7.7 Hz, NH (couples to signal at 3.88)), 7.23 (5 H, m, Ar), 7.37 (5 H, m, Ar), 7.71 (1 H, t, $J$ = 5.9 Hz, NH (couples to signal at 2.99)), 7.76 (1 H, t, $J$ = 5.4 Hz, NH (couples to signal at 2.99)), 7.86 (1 H, d, $J$ = 7.8 Hz, NH (couples to signal at 4.16)), 7.92 (4 H, m, $\underline{H}NCH_2CH_2N\underline{H}$ + 2 × Gly-NH), 8.03 (1 H, d, $J$ = 8.3 Hz, Phe α-NH), 8.13 (1 H, d, $J$ = 7.8 Hz, Glu α-NH)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.25 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.37 (13 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.50 (6 H, m, Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.62 (2 H, m, Leu γ-H + Glu β-H), 1.83 (1 H , m, Glu β-H), 2.07 (2 H, t, $J$ = 7.8 Hz, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76 (1 H, dd, $J$ = 15, 9 Hz, Phe β-$H_2$), 2.89 (3 H, s, Sar-$H_3$), 2.99 (5 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.64 (2 H, m, Gly-$H_2$), 3.72 (2 H, d, $J$ = 5.4 Hz, Gly-$H_2$) 3.83 (2 H, s, Sar-$H_2$), 3.88 (1 H, m, Leu α-H or Lys α-H), 4.16 (1 H, m, Leu α-H or Lys α-H), 4.23 (1 H, m, Glu α-H), 4.54 (1 H, m, Phe α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.08 (2 H, s, Ar-$CH_2$), 6.92 (1 H, d, $J$ = 7.7 Hz, NH (couples to signal at 3.88)), 7.23 (5 H, m, Ar), 7.37 (5 H, m, Ar), 7.71 (1 H, t, $J$ = 5.9 Hz, NH (couples to signal at 2.99)), 7.76 (1 H, t, $J$ = 5.4 Hz, NH (couples to signal at 2.99)), 7.86 (1 H, d, $J$ = 7.8 Hz, NH (couples to signal at 4.16)), 7.92 (4 H, m, $\underline{H}NCH_2CH_2N\underline{H}$ + 2 × Gly-NH), 8.03 (1 H, d, $J$ = 8.3 Hz, Phe α-NH), 8.13 (1 H, d, $J$ = 7.8 Hz, Glu α-NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.27 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.38 (13 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.54 (6 H, m, Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.65 (1 H, m, Leu γ-H), 1.74 (1 H, m, Glu β-H), 1.87 (1 H, m, Glu β-H), 2.10 (2 H, t, $J$ = 7.6 Hz, Glu γ-$H_2$), 2.26 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.82 (1 H, dd, $J$ = 14, 9 Hz, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$),

3.05 (5 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.58 (3 H, s, $OCH_3$), 3.64 (2 H, m, Gly-$H_2$), 3.72 (2 H, d, $J$ = 5.9 Hz, Gly-$H_2$) 3.83 (2 H, s, Sar-$H_2$), 3.94 (1 H, m, Leu α-H or Lys α-H), 4.16 (1 H, m, Leu α-H or Lys α-H), 4.24 (1 H, m, Glu α-H), 4.54 (1 H, m, Phe α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.22 (5 H, m, Ar), 7.33 (5 H, m, Ar), 7.47 (1 H, t, $J$ = 4.1 Hz, NH) 7.68 (3 H, m, 3 × NH), 7.81 (3 H, m, 3 × NH)

T.L.C. (C) Rf = 0.19

**$N^{\alpha}$-(N-(N-(N-(N-($N^{\delta}$-(6-Methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (235)**

Hydrogen chloride was bubbled through a solution of $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(1,1-dimethylethoxycarbonyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (9.38 g, 7.2 mmol) in $CH_2Cl_2$ (200 ml) and MeOH (100 ml) for 40 min. The solvents and excess hydrogen chloride were evaporated to give $N^{\alpha}$-(N-(N-(N-(N-($N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (8.93 g, quantitative) as a pale yellow foam.

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.88 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.28 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.42 (6 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.55 (6 H, m, Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.61 (2 H, m, Leu γ-H + Glu β-$H_2$), 1.74 (1 H , m, Glu β-$H_2$), 2.18 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.84 (1 H, m, Phe β-$H_2$), 2.88 (3 H, s, Sar-$H_3$), 3.04 (5 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.15 (4 H, br, $NCH_2CH_2N$), 3.58 (3 H, s, $OCH_3$), 3.75 (4 H, m, 2 × Gly-$H_2$), 3.90 (2 H, s, Sar-$H_2$), 4.15 (2 H, m, 2 × α-H), 4.28 (1 H, m, α-H), 4.55 (1 H, m, α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.06 (2 H, s, Ar-$CH_2$), 7.23 (5 H, m, Ar), 7.33 (5 H, m, Ar), 7.67 (3 H, br 3 × NH), 7.76 (1 H, br, NH), 7.84 (1 H, m, NH), 7.99 (1 H, d, $J$ = 7.9 Hz, NH), 8.07 (1 H, d, $J$ = 8.3 Hz, NH), 8.27 (4 H, m, $NH_3^+$ + NH), 8.55 (1 H, m, NH).

T.L.C. (C) Rf = 0.12

MS (FAB-) 1200 (M + Cl)

**$N^{\alpha}$-(N-(N-(N-(N-($N^{\delta}$-(6-Methoxy-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (236)**

N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (1.04 g, 2.7 mmol) was added to a stirred solution of $N^{\alpha}$-(N-(N-(N-(N-($N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)-glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (2.84 g, 2.2 mmol), N,N-diisopropylethylamine (3.20 g, 24.8 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (30 ml) and dimethylformamide (10 ml). After 18 h, a further portion of N-(phenylmethoxy-carbonyl)sarcosine pentafluorophenyl ester (1.04 g, 2.7 mmol) was added and stirring continued for 2 d. The solvents were evaporated and the residue was dissolved in EtOAc / MeOH (1:1). The solution was washed with cold 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1 then $CH_2Cl_2$ / MeOH 15:1) gave $N^{\alpha}$-(N-(N-(N-(N-($N^{\delta}$-(6-methoxy-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)-phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (1.25 g, 40%) as a cream solid. Mp 181-183°C

$^1$H NMR ($(CD_3)_2SO$) 20°C (COSY 90)

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.22 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.48 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.63 (3 H, m, Leu β-H + Leu γ-$H_2$), 1.73 (1 H, m, Glu β-$H_2$), 1.85 (1 H, m, Glu β-$H_2$), 2.07 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.76 (2 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 3.01 (4 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.56 (3 H, s, $OCH_3$), 3.61 (1 H, m, Gly-$H_2$),

3.72 (3 H, m, Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.88 (2 H, s, Sar-$H_2$), 4.13 (1 H, m, Leu α-H or Lys α-H or Glu α-H), 4.26 (2 H, m, Leu α-H or Lys α-H or Glu α-H), 4.53 (1 H, m, Phe α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$H_2$), 5.09 (2 H, s, Ar-$H_2$), 7.30 (15 H, m, Ar + Phe-Ar), 7.67 (1 H, t, $J$ = 5.8 Hz, NH (couples to signal at 3.01)), 7.75 (1 H, m, NH (couples to signal at 3.01)), 7.89 (1 H, d, $J$ = 7.8 Hz, NH (couples to signal at 4.13)), 7.97 (2 H, br, $\underline{H}NCH_2CH_2N\underline{H}$), 8.05 (1 H, t, $J$ = 9.3 Hz, Phe NH), 8.16 (4 H, m, 2 × Gly NH + 2 × NH (couples to signal at 4.26).

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.22 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.48 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.63 (3 H, m, Leu β-H + Leu γ-$H_2$), 1.73 (1 H, m, Glu β-$H_2$), 1.85 (1 H, m, Glu β-$H_2$), 2.07 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.76 (2 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 3.01 (4 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.56 (3 H, s, $OCH_3$), 3.61 (1 H, m, Gly-$H_2$), 3.72 (3 H, m, Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 4.13 (1 H, m, Leu α-H or Lys α-H or Glu α-H), 4.26 (2 H, m, Leu α-H or Lys α-H or Glu α-H), 4.53 (1 H, m, Phe α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.05 (2 H, s, Ar-$H_2$), 5.08 (2 H, s, Ar-$H_2$), 7.30 (15 H, m, Ar + Phe-Ar), 7.67 (1 H, t, $J$ = 5.8 Hz, NH (couples to signal at 3.01)), 7.75 (1 H, m, NH (couples to signal at 3.01)), 7.89 (1 H, d, $J$ = 7.8 Hz, NH (couples to signal at 4.13)), 7.97 (2 H, br, $\underline{H}NCH_2CH_2N\underline{H}$), 8.05 (1 H, t, $J$ = 9.3 Hz, Phe NH), 8.16 (4 H, m, 2 × Gly NH + 2 × NH (couples to signal at 4.26)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.83 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.26 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.42 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.52 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.64 (3 H, m, Leu β-H + Leu γ-$H_2$), 1.77 (1 H, m, Glu β-$H_2$), 1.91 (1 H, m, Glu β-$H_2$), 2.10 (2 H, t, $J$ = 7.6 Hz Glu γ-$H_2$), 2.26 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.76 (1 H, m, Phe β-$H_2$), 2.87 (3 H, s, Sar-$H_3$), 2.88 (3 H, s, Sar-$H_3$), 3.05 (5 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.71 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.92 (2 H, br, Sar-$H_2$), 4.13 (1 H, m, Leu α-H or Lys α-H or Glu α-H or Phe), 4.26 (2 H, m, Leu α-H or Lys α-H or Glu α-H or Phe), 4.53 (1 H, m, Leu α-H or Lys α-H or Glu α-H or Phe), 4.74 (2 H, s, $CH_2CCl_3$), 5.04 (2 H, s, Ar-$H_2$), 5.06 (2 H, br, Ar-$H_2$), 7.30 (15 H, m, Ar + Phe-Ar), 7.46 (1 H, m, NH), 7.58 (1 H, d, $J$ = 7.9 Hz, NH), 7.66 (2 H, br, $\underline{H}NCH_2CH_2N\underline{H}$), 8.05 (1 H, br, NH), 7.80 (3 H, m, 3 × NH), 7.89 (1 H, m, NH), 7.95 (1 H, d, $J$ = 7.3 Hz, NH)

T.L.C. (C) Rf = 0.54

Acc. Mass 1403.5544 (M + H) ($C_{64}H_{90}{}^{35}Cl_3N_{12}O_{17}$ requires 1403.5612)

**$N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (243)**

Zinc powder (4.34 g, 66.39 mmol) was added to a solution of $N^{\alpha}$-(N-(phenylmethoxy-carbonyl)sarcosyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide) (1.25 g, 1.65 mmol) in 70% MeOH / water (10 ml) and boiled under reflux for 7 h. The mixture was filtered and the solvents were evaporated to give $N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (953 mg, 97%) as a colourless glass.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 1.23 (2 H, m, Lys β,γ,δ-$H_2$), 1.50 (2 H, m, Lys β,γ,δ-$H_2$), 1.62 (2 H, m, Lys β,γ,δ-$H_2$), 2.73 (2 H, m, Lys ε-$H_2$), 2.85 (2 H, s, Sar-$H_3$), 2.86 (2 H, br, Sar-$H_3$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.84 (2 H, br, Sar-$H_2$), 3.93 (2 H, br, Sar-$H_2$), 4.20 (1 H, m, Lys α-H), 5.03 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.34 (10 H, m, 2 × Ar), 8.04-8.25 (4 H, m, 4 × NH)

δ 1.23 (2 H, m, Lys β,γ,δ-$H_2$), 1.50 (2 H, m, Lys β,γ,δ-$H_2$), 1.62 (2 H, m, Lys β,γ,δ-$H_2$), 2.73 (2 H, m, Lys ε-$H_2$), 2.86 (2 H, s, Sar-$H_3$), 2.89 (2 H, br, Sar-$H_3$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.84 (2 H, br, Sar-$H_2$), 3.93 (2 H, br, Sar-$H_2$), 4.20 (1 H, m, Lys α-H), 5.04 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.34 (10 H, m, 2 × Ar), 8.04-8.25 (4 H, m, 4 × NH).

T.L.C. (C) Rf = 0.15

Acc. Mass 599.3176 (M + H) ($C_{30}H_{43}N_6O_7$ requires 599.3193)

**Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (244)**

6-(1,1-Dimethylethoxycarbonylamino)hexanoic acid (13.0 g, 56.3 mmol) in EtOAc (100 ml) was cooled to 0°C. DCC (11.6 g, 56.3 mmol) and pentafluorophenol (10.35 g, 56.3 mmol) were added and the suspension stirred for 24 h. The suspension was filtered. The

$^1$H NMR ($(CD_3)_2SO$)

δ 1.19 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (11 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.45 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.01 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.85 (3 H, s, Sar-$H_3$), 2.88 (3 H, br, Sar-$H_3$), 2.96 (4 H, m, Lys ε-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.13 (1 H, m, Lys α-H), 5.02 (2 H, s, Ar-$CH_2$), 5.07 (2 H, br, Ar-$CH_2$), 6.78 (1 H, m, NH), 7.36 (10 H, 2 × Ar), 7.73 (1 H, m, NH), 7.96 (3 H, br, 3 × NH)

δ 1.19 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (11 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.45 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 2.01 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.86 (3 H, s, Sar-$H_3$), 2.88 (3 H, br, Sar-$H_3$), 2.96 (4 H, m, Lys ε-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.13 (1 H, m, Lys α-H), 5.04 (2 H, s, Ar-$CH_2$), 5.07 (2 H, br, Ar-$CH_2$), 6.78 (1 H, m, NH), 7.36 (10 H, 2 × Ar), 7.73 (1 H, m, NH), 7.96 (3 H, br, 3 × NH)

T.L.C. (C) Rf = 0.41

MS (FAB+) 712 (M - Boc)

**$N^{\varepsilon}$-(6-Aminohexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (246)**

$N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.18 g, 2.7 mmol) in $CH_2Cl_2$ (20 ml) and MeOH (5 ml) was treated with hydrogen chloride for 30 min. The solvents and excess reagent were evaporated to give $N^{\varepsilon}$-(6-aminohexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide hydrochloride (1.93 g, 96%) as a pale foam.
This material was carried forward without characterisation.

**$N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (247)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (520 mg, 0.67 mmol) was added to $N^{\varepsilon}$-(6-aminohexanoyl)-$N^{\alpha}$-(N-(phenylmethoxy-carbonyl)sarcosyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (385 mg, 0.53 mmol), N,N-diisopropylethylamine (205 mg, 1.59 mmol) and DMAP (10 mg) in $CHCl_3$ (20 ml). The solution was stirred for 24 h and the solvents were evaporated. Chromatography ($CHCl_3$ then $CHCl_3$ / MeOH 80 : 1 then $CHCl_3$ / MeOH 40 : 1 then $CHCl_3$ / MeOH 20 : 1) yielded $N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)-hexanoyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (593 mg, 82%) as a purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.78 (2 H, s, *21,23*-$H_2$), 1.2-1.7 (12 H, m, Lys β,γ,δ-$H_6$ + $NCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2$), 2.12 (2 H, br, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.93 (3 H, s, Sar-$H_3$), 2.99 (3 H, s, Sar-$H_3$), 3.0-3.3 (8 H, m, Lys ε-$H_2$ + $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + $NCH_2CH_2N$), 3.77 (2 H, m, Sar-$H_2$), 3.86 (2 H, m, Sar-$H_2$), 4.36 (1 H, m, Lys α-H), 5.06 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 5.75 (1 H, br, NH), 5.83 (1 H, br, NH), 6.39 (1 H, br, NH), 6.57 (1 H, br, NH), 6.94 (1 H, br, NH), 7.24 (10 H, br, 2 × Ar), 7.37 (1 H, br, NH)

δ -2.78 (2 H, s, *21,23*-$H_2$), 1.2-1.7 (12 H, m, Lys β,γ,δ-$H_6$ + $NCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2$), 2.12 (2 H, br, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.99 (3 H, s, Sar-$H_3$), 3.00 (3 H, s, Sar-$H_3$), 3.0-3.3 (8 H, m, Lys ε-$H_2$ + $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + $NCH_2CH_2N$), 3.77 (2 H, m, Sar-$H_2$), 3.86 (2 H, m, Sar-$H_2$), 4.36 (1 H, m, Lys α-H), 5.07 (4 H, s, 2 × Ar-$CH_2$), 5.75 (1 H, br, NH), 5.83 (1 H, br, NH), 6.39 (1 H, br, NH), 6.57 (1 H, br, NH), 6.94 (1 H, br, NH), 7.24 (10 H, br, 2 × Ar), 7.37 (1 H, br, NH)

T.L.C. (C) Rf = 0.32

**N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (248)**

Zinc powder (5.5 g, 86.5 mmol) was added to N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxy-carbonyl)sarcosyl)-N$^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.50 g, 2.58 mmol) in 70% MeOH / water (20 ml) and the mixture boiled under reflux for 18 h. The mixture was filtered and the solvents evaporated to yield N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxy-carbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (2.04 g 94%) as a white foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.83 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.25 (2 H, m, Lys β,γ,δ-$H_2$), 1.52 (5 H, m, Leu β-$H_2$ + Leu γ–H + Lys β,γ,δ–$H_2$), 1.61 (2 H, m, Lys β,γ,δ–$H_2$), 2.69 (2 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.86 (3 H, s, Sar-$H_3$), 3.05 (2 H, m, Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.63 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, 2 × α-H), 4.54 (1 H, m, α-H), 5.01 (2 H, s, Ar-$H_2$), 5.03 (2 H, s, Ar-$H_2$), 7.35 (15 H, m, Ar + Phe-Ar), 7.83 (1 H, br, NH), 8.10 (3 H, br, 3 × NH), 8.25 (4 H, m, 4 × NH)

δ 0.83 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.25 (2 H, m, Lys β,γ,δ-$H_2$), 1.52 (5 H, m, Leu β-$H_2$ + Leu γ–H + Lys β,γ,δ–$H_2$), 1.61 (2 H, m, Lys β,γ,δ–$H_2$), 2.69 (2 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.88 (3 H, s, Sar-$H_3$), 3.05 (2 H, m, Lys ε-$H_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.63 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, 2 × α-H), 4.54 (1 H, m, α-H), 5.03 (2 H, s, Ar-$H_2$), 5.07 (2 H, s, Ar-$H_2$), 7.35 (15 H, m, Ar + Phe-Ar), 7.83 (1 H, br, NH), 8.10 (3 H, br, 3 × NH), 8.25 (4 H, m, 4 × NH)

T.L.C. (C) Rf = 0.18

Acc. Mass 972.5094 (M + H) ($C_{49}H_{68}N_{10}O_{11}$ requires 972.5069)

**N-(N-(N-(N-($N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (249)**

Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (2.6 g, 6.57 mmol) was added to N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.15 g, 2.19 mmol), N,N-diisopropylethylamine (850 mg, 6.56 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (20 ml) and DMF (10 ml). The solution was stirred for 5 d. The solvents were evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1) gave N-(N-(N-(N-($N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (1.43 g, 55%) as a pale yellow glass.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.82 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.2-1.7 (24 H, m, Bu-t + Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.01 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76-3.04 (2 H, m, Phe β-$H_2$), 2.83 (2 H, s, Sar-$H_3$), 2.86 (2 H, br, Sar-$H_3$), 3.17 (8 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$), 3.59 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.24 (2 H, m, 2 × α–H), 4.53 (1 H, m, α-H), 5.01 (2 H, br, Ar-$CH_2$), 5.03 (2 H, s, Ar-$CH_2$), 6.76 (1 H, m, NH), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.75 (1 H, m, NH), 7.84 (1 H, m, NH), 8.04 (2 H, m, 2 × NH), 8.20 (3 H, m, 3 × NH), 8.97 (1 H, m, NH)

δ 0.82 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.2-1.7 (24 H, m, Bu-t + Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.01 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76-3.04 (2 H, m, Phe β-$H_2$), 2.86 (2 H, s, Sar-$H_3$), 2.88 (2 H, br, Sar-$H_3$), 3.17 (8 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$), 3.59 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.24 (2 H, m, 2 × α-H), 4.53 (1 H, m, α-H), 5.03 (2 H, br, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 6.76 (1 H, m, NH), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.75 (1 H, m, NH), 7.84 (1 H, m, NH), 8.04 (2 H, m, 2 × NH), 8.20 (3 H, m, 3 × NH), 8.97 (1 H, m, NH)

T.L.C. (C) Rf = 0.31

**N-(N-(N-(N-(N$^{\varepsilon}$-(6-Aminohexanoyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)-glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (250)**

Hydrogen chloride was bubbled through N-(N-(N-(N-(N$^{\varepsilon}$-(6-(1,1-dimethylethoxy-carbonylamino)hexanoyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenyl-alanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.40 g, 1.17 mmol) in $CH_2Cl_2$ (30 ml) for 35 min. MeOH (5 ml) was added and the solvents and excess reagent were evaporated to give N-(N-(N-(N-(N$^{\varepsilon}$-(6-amino-hexanoyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.33 g, quantitative) as a white foam.
This material was carried forward without characterisation.

**N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-N$^{\varepsilon}$-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylaminocarbonyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (251)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (2.54 g, 3.26 mmol) was added to N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (2.04 g, 2.07 mmol), N,N-diisopropylethylamine (1.34 g, 10.35 mmol), DMAP (20 mg) in $CHCl_3$ (30 ml) and DMF (10 ml). The solution was stirred for 24 h and the solvents were then evaporated. Chromatography ($CHCl_3$ / MeOH 80:1 then $CHCl_3$ / MeOH 40:1 then $CHCl_3$ / MeOH 20:1 then $CHCl_3$ / MeOH 10:1) yielded N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-N$^{\varepsilon}$-(4-(10,15,20-tri-phenyl-21*H*,23*H*-porphin-5-yl)phenylaminocarbonyl)lysyl)glycyl)phenylalanyl)leucyl)-glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.29 g, 98%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -2.90 (2 H, s, *21,23*H), 0.83 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 1.2-1.8 (9 H, m, Lys β,γ,δ–$H_2$ + Leu γ-H + Leu β-$H_2$), 2.79 (1 H, m, Phe β-$H_2$), 2.82 (3 H, s, Sar-$H_3$), 2.86 (3 H, br, Sar-$H_3$), 3.16 (7 H, br, $NCH_2CH_2N$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.68 (4 H, m, 2 × Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.98 (2 H, br, Sar-$H_2$), 4.32 (2 H, m, 2 × α-H), 4.59 (1 H, m, α-H), 5.00 (2 H, s, Ar-$CH_2$), 5.04 (2 H, s, Ar-$CH_2$), 6.35 (1 H, br, NH), 7.29 (15 H, m, 2 × Ar + Phe-Ar), 7.84 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 8.07 (2 H, d, *J* = 8.2 Hz, disubstituted aromatic 3,5-$H_2$), 8.19 (11 H, m, 3 × Ph 2,6-$H_2$ + 5 × NH), 8.75-9.00 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

δ -2.90 (2 H, s, *21,23*H), 0.83 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 1.2-1.8 (9 H, m, Lys β,γ,δ–$H_2$ + Leu γ-H + Leu β-$H_2$), 2.79 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.89 (3 H, br, Sar-$H_3$), 3.16 (7 H, br, $NCH_2CH_2N$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.68 (4 H, m, 2 × Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.98 (2 H, br, Sar-$H_2$), 4.32 (2 H, m, 2 × α-H), 4.59 (1 H, m, α-H), 5.04 (4 H, s, 2 × Ar-$CH_2$), 6.35 (1 H, br, NH), 7.29 (15 H, m, 2 × Ar + Phe-Ar), 7.84 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 8.07 (2 H, d, *J* = 8.2 Hz, disubstituted aromatic 3,5-$H_2$), 8.19 (11 H, m, 3 × Ph 2,6-$H_2$ + 5 × NH), 8.75-9.00 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

T.L.C. (C) Rf = 0.42

MS (FAB+) 1629 (M + H)

**N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-N$^{\varepsilon}$-(6-(N'-(4-(10,15,20-tri-phenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (252)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (1.41 g, 1.81 mmol) was added to N-(N-(N-(N-(N$^{\varepsilon}$-(6-aminohexanoyl)-N$^{\alpha}$-(N-(phenyl-methoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.28 g, 1.13 mmol), N,N-diisopropylethylamine (730 mg, 5.66 mmol) and DMAP (10 mg) in $CHCl_3$ (20 ml) and DMF (10 ml). The solution was stirred for 48 h and the solvents were evaporated. Chromatography (a) ($CHCl_3$ / MeOH 100:1 then $CHCl_3$ / MeOH 80:1 then $CHCl_3$ /

MeOH 60:1 then $CHCl_3$ / MeOH 1:1) and (b) ( $CHCl_3$ / MeOH 9:1) yielded N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.25 g, 64%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ -2.9 (2 H, s, *21,23*H), 0.82 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.2-1.6 (15 H, m, Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.10 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.78 (1 H, m, Phe β-$H_2$), 2.84 (2 H, s, Sar-$H_3$), 2.85 (2 H, s, Sar-$H_3$), 3.12 (9 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + Phe β-$H_2$), 3.55-3.78 (4 H, m, 2 × Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.24 (2 H, m, 2 × α-H), 4.55 (1 H, m, α-H), 5.01 (2 H, br, Ar-$CH_2$), 5.05 (2 H, s, Ar-$CH_2$), 7.29 (15 H, m, 2 × Ar + Phe-Ar), 7.82 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 8.06 (2 H, d, *J* = 8.4 Hz, disubstituted aromatic 3,5-$H_2$), 8.23 (11 H, m, 3 × Ph 2,6-$H_2$ + 5 × NH), 8.80-9.00 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

δ -2.9 (2 H, s, *21,23*H), 0.82 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.87 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.2-1.6 (15 H, m, Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.10 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.78 (1 H, m, Phe β-$H_2$), 2.85 (2 H, s, Sar-$H_3$), 2.87 (2 H, s, Sar-$H_3$), 3.12 (9 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + Phe β-$H_2$), 3.55-3.78 (4 H, m, 2 × Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.24 (2 H, m, 2 × α-H), 4.55 (1 H, m, α-H), 5.01 (2 H, br, Ar-$CH_2$), 5.05 (2 H, s, Ar-$CH_2$), 7.29 (15 H, m, 2 × Ar + Phe-Ar), 7.82 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 8.06 (2 H, d, *J* = 8.4 Hz, disubstituted aromatic 3,5-$H_2$), 8.23 (11 H, m, 3 × Ph 2,6-$H_2$ + 5 × NH), 8.80-9.00 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

T.L.C. (C) Rf = 0.48

MS (FAB-) 1740 (M - H)

**$N^{\alpha}$-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (253)**

Zinc powder (6.5 g, 99.4 mmol) was added to $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxy-carbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.04 g, 2.68 mmol) in 70% MeOH / water (20 ml). The mixture was boiled under reflux for 24 h and was then filtered. The solvents were evaporated to give $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxy-carbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (2.50 g, 95%) as a cream foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.82 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.29 (2 H, m, Lys β,γ,δ–$H_2$), 1.52 (5 H, m, Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ–$H_2$), 1.64 (2 H, m, Lys β,γ,δ–$H_2$), 2.72 (2 H, m, Lys ε-$H_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 3.02 (1 H, br, Phe β-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.61 (2 H, m, Gly-$H_2$), 3.73 (2 H, m, Gly-$H_2$), 3.73 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.24 (1 H, m, α-H), 4.53 (1 H, m, α-H), 5.01 (2 H, s, Ar-$H_2$), 5.07 (2 H, br, Ar-$H_2$), 7.30 (15 H, m, Ar + Phe-Ar), 8.03 (4 H, br, 4 × NH), 8.20 (4 H, m, 4 × NH)

δ 0.82 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.29 (2 H, m, Lys β,γ,δ–$H_2$), 1.52 (5 H, m, Leu β-$H_2$ + Leu γ-H + Lys β,γ,δ–$H_2$), 1.64 (2 H, m, Lys β,γ,δ–$H_2$), 2.72 (2 H, m, Lys ε-$H_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.88 (3 H, s, Sar-$H_3$), 3.02 (1 H, br, Phe β-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.61 (2 H, m, Gly-$H_2$), 3.73 (2 H, m, Gly-$H_2$), 3.73 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.24 (1 H, m, α-H), 4.53 (1 H, m, α-H), 5.03 (2 H, s, Ar-$H_2$), 5.07 (2 H, br, Ar-$H_2$), 7.30 (15 H, m, Ar + Phe-Ar), 8.03 (4 H, br, 4 × NH), 8.20 (4 H, m, 4 × NH)

T.L.C. (C) Rf = 0.18

**$N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (254)**

Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (2.90 g, 7.2 mmol) was added to $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.41 g, 2.45 mmol), N,N-diisopropylethylamine (950 mg, 7.2 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (20 ml) and DMF (10 ml). The solution was stirred for 5 d and the solvents were evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1) yielded $N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)-sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (1.35 g, 51%) as a cream foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.21 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (13 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.47 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.63 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.00 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76 (2 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.86 (3 H, s, Sar-$H_3$), 2.99 (4 H, m, Lys ε-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.62 (2 H, m, Gly-$H_2$), 3.73 (2 H, m, Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.12 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.53 (1 H, m, α-H), 5.02 (2 H, s, Ar-$H_2$), 5.07 (2 H, br, Ar-$H_2$), 6.76 (1 H, m, NH), 7.30 (15 H, m, Ar + Phe-Ar), 7.72 (1 H, m, NH), 7.80 (1 H, br, NH), 7.98 (3 H, br, 3 × NH), 8.14 (3 H, m, 3 × NH)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.21 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (13 H, m, Bu-t + Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.47 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.63 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.00 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76 (2 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 2.99 (4 H, m, Lys ε-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.62 (2 H, m, Gly-$H_2$), 3.73 (2 H, m, Gly-$H_2$), 3.82 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.12 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.53 (1 H, m, α-H), 5.03 (2 H, s, Ar-$H_2$), 5.07 (2 H, br, Ar-$H_2$), 6.76 (1 H, m, NH), 7.30 (15 H, m, Ar + Phe-Ar), 7.72 (1 H, m, NH), 7.80 (1 H, br, NH), 7.98 (3 H, br, 3 × NH), 8.14 (3 H, m, 3 × NH).

T.L.C. (C) Rf = 0.33

**$N^{\varepsilon}$-(6-Aminohexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)-glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (255)**

$N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenyl-methoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (1.35 g, 1.13 mmol) in $CH_2Cl_2$ (30 ml) was treated with hydrogen chloride for 40 min. Methanol (5 ml) was added and the solvents and excess reagent evaporated to give $N^{\varepsilon}$-(6-aminohexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.28 g, quantitative) as an off white foam.

This material was carried forward without characterisation.

**$N^{\alpha}$-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylaminocarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (256)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (2.14 g, 2.74 mmol) was added to $N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.71 g, 1.74 mmol), N,N-diisopropylethylamine (1.13 g, 8.70 mmol) and DMAP (20 mg) in $CHCl_3$ (30 ml) and DMF (10 ml). The solution was stirred for 24 h. The solvents were evaporated. Chromatography ($CHCl_3$ then $CHCl_3$ / MeOH 60 : 1 then $CHCl_3$ / MeOH 40 : 1 then $CHCl_3$ / MeOH 20 : 1 then $CHCl_3$ / MeOH 10 : 1) gave $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(4-(10,15,20-tri-

phenyl-21*H*,23*H*-porphin-5-yl)phenylaminocarbonyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (2.37 g, 84%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -2.90 (2 H, s, *21,23*-H), 0.84 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 1.18-1.80 (9 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.72 (1 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 3.16 (7 H, m, $NCH_2CH_2N$ + Phe β-$H_2$ + Lys ε-$H_2$), 3.55-3.72 (4 H, m, 2 × Gly-$H_2$), 3.86 (2 H, s, Sar-$H_2$), 3.87 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, 2 × α-H), 4.58 (1 H, m, α-H), 5.00 (2 H, s, Ar-$CH_2$), 5.04 (2 H, br, Ar-$CH_2$), 6.36 (1 H, t, *J* = 6 Hz, NH), 7.28 (15 H, m, 2 × Ar + Phe-Ar), 7.84 (11 H, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.95-8.25 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 5 × NH), 8.8-9.0 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

δ -2.90 (2 H, s, *21,23*-H), 0.84 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 0.88 (3 H, d, *J* = 6.3 Hz, Leu-$H_3$), 1.18-1.80 (9 H, m, Lys β,γ,δ-$H_2$ + Leu γ-H + Leu β-$H_2$), 2.72 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.90 (3 H, s, Sar-$H_3$), 3.16 (7 H, m, $NCH_2CH_2N$ + Phe β-$H_2$ + Lys ε-$H_2$), 3.55-3.72 (4 H, m, 2 × Gly-$H_2$), 3.86 (2 H, s, Sar-$H_2$), 3.87 (2 H, br, Sar-$H_2$), 4.25 (2 H, m, 2 × α-H), 4.58 (1 H, m, α-H), 5.04 (2 H, s, Ar-$CH_2$), 5.06 (2 H, br, Ar-$CH_2$), 6.36 (1 H, t, *J* = 6 Hz, NH), 7.28 (15 H, m, 2 × Ar + Phe-Ar), 7.84 (11 H, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.95-8.25 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 5 × NH), 8.8-9.0 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

T.L.C. (C) Rf = 0.48

MS (FAB+) 1629 (M + H)

**$N^{\alpha}$-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)-glycyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)-hexanoyl)lysine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (257)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (1.36 g, 1.74 mmol) was added to $N^{\varepsilon}$-(6-aminohexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-(N-(phenyl-methoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.23 g, 1.09 mmol), N,N-

diisopropylethylamine (704 mg, 5.45 mmol) and DMAP (10 mg) in $CHCl_3$ (30 ml) and DMF (10 ml). The solution was stirred for 48 h, then the solvents were evaporated. Chromatography (a) ($CHCl_3$ / MeOH 80 : 1 then $CHCl_3$ / MeOH 60 : 1 then $CHCl_3$ / MeOH 40 : 1 then $CHCl_3$ / MeOH 30:1 then $CHCl_3$ / MeOH 25:1 then $CHCl_3$ / MeOH 15 : 1 then $CHCl_3$ / MeOH 12 : 1 then $CHCl_3$ / MeOH 10 : 1 then $CHCl_3$ / MeOH 1 : 1) and (b) ($CHCl_3$ / MeOH 9 : 1) gave $N^{\alpha}$-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)-sarcosyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (1.52 g, 80%) as a purple glass.

$^1H$ NMR ($(CD_3)_2SO$) 20°C

δ -2.91 (2 H, s, *21,23*-H), 0.83 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.35-160 (11 H, m, 2 × Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$ + Leu γ-H + Leu β-$H_2$), 2.10 (2 H. m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.80 (1 H, m, Phe β-$H_2$), 2.82 (3 H, s, Sar-$H_3$), 2.85 (3 H, s, Sar-$H_3$), 3.00-3.10 (9 H, m, Lys ε–$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + $NCH_2CH_2N$ + Phe β-$H_2$), 3.55-3.75 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.87 (2 H, br, Sar-$H_2$), 4.18 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.59 (1 H, m, α-H), 5.00 (2 H, s, Ar-$H_2$), 5.02 (2 H, br, Ar-$H_2$), 6.36 (1 H, t, *J* = 6 Hz, NH), 7.28 (15 H, m, 2 × Ar + Phe-Ar), 7.83 (11 H, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.96-8.24 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 5 × NH), 8.75-9.0 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

δ -2.91 (2 H, s, *21,23*-H), 0.83 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, *J* = 6.2 Hz, Leu-$H_3$), 1.35-160 (11 H, m, 2 × Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$ + Leu γ-H + Leu β-$H_2$), 2.10 (2 H. m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.80 (1 H, m, Phe β-$H_2$), 2.85 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 3.00-3.10 (9 H, m, Lys ε–$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + $NCH_2CH_2N$ + Phe β-$H_2$), 3.55-3.75 (4 H, m, 2 × Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.87 (2 H, br, Sar-$H_2$), 4.18 (1 H, m, α-H), 4.22 (1 H, m, α-H), 4.59 (1 H, m, α-H), 5.02 (2 H, s, Ar-$H_2$), 5.05 (2 H, br, Ar-$H_2$), 6.36 (1 H, t, *J* = 6 Hz, NH), 7.28 (15 H, m, 2 × Ar + Phe-Ar), 7.83 (11 H, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.96-8.24 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 5 × NH), 8.75-9.0 (11 H, m, *2,3,7,8,12,13,17,18*-$H_8$ + 3 × NH)

T.L.C. (C) Rf = 0.42

MS (FAB+) 1742 (M + H)

**$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (258)**

Zinc powder (4.5 g, 68.8 mmol) was added to a suspension of $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.5 g, 1.78 mmol) in 70% MeOH / water (20 ml). The mixture was boiled under reflux for 26 h. The mixture was filtered and the solvents were evaporated to give $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.98 g, 89%) as a pale foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.82 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.24 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.33 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.63 (3 H, m, Leu β-$H_2$ + Leu γ-H + Glu β-$H_2$), 2.06 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.74 (2 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.86 (3 H, br, Sar-$H_3$), 3.00 (4 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.63 (1 H, m, Gly-$H_2$), 3.72 (3 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.21 (3 H, m, 3 × α-H), 4.51 (1 H, m, α-H), 5.01 (2 H, s, Ar-$H_2$), 5.04 (2 H, br, Ar-$H_2$), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.77 (1 H, br, NH), 8.01 (5 H, m, 5 × NH), 8.19 (4 H, m, 4 × NH).

δ 0.82 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.24 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.33 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.63 (3 H, m, Leu β-$H_2$ + Leu γ-H + Glu β-$H_2$), 2.06 (2 H, m, Glu γ-$H_2$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.74 (2 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.89 (3 H, br, Sar-$H_3$), 3.00 (4 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.63 (1 H, m, Gly-$H_2$), 3.72 (3 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.21 (3 H, m, 3 × α-H), 4.51 (1 H, m, α-H), 5.04 (2 H, s, Ar-$H_2$), 5.08 (2 H, br, Ar-$H_2$), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.77 (1 H, br, NH), 8.01 (5 H, m, 5 × NH), 8.19 (4 H, m, 4 × NH).

T.L.C. (C) Rf = 0.17

**$N^{\varepsilon}$-(6-(1,1-Dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)-phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)-ethyl)amide (259)**

Pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (896 mg, 2.27 mmol) was added to $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.89 g, 1.51 mmol), N,N-dimethylethylamine (530 mg, 6.08 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (15 ml) and DMF (15 ml). The solution was stirred for 24 h then pentafluorophenyl 6-(1,1-dimethylethoxycarbonylamino)hexanoate (896 mg, 2.27 mmol) was added. Stirring was continued for 3d and the solvents were evaporated. Chromatography ($CH_2Cl_2$ / MeOH 20:1 then MeOH) yielded $N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonylamino)hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (1.95 g, 89%) as a buff foam.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.82 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 1.22 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.36 (17 H, m, Bu-t + Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + 2 × $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.50 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.59-1.92 (3 H, m, Leu γ-H + Glu β-$H_2$), 2.04 (4 H, m, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.27 (2 H. t. $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.76-3.18 (18 H, m, Sar-$H_3$ + Phe β-$H_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + 2 × $NC\underline{H}_2CH_2CH_2CH_2CH_2$), 3.57 (3 H, s, $OCH_3$), 3.65 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 3.92 (2 H, br, Sar-$H_2$), 4.20 (3H, m, 3 × α-H), 4.49 (1 H, m, 1 × α-H), 5.01 (2 H, s, Ar-$H_2$), 5.07 (2 H, s, Ar-$H_2$), 6.78 (1 H, t, $J$ = 6.7 Hz, NH), 7.22 (5 H, m, Ar), 7.36 (10 H, m, Ar), 7.78 (1 H, d, $J$ = 6.7 Hz, NH), 7.91 (1 H, d, $J$ = 7.8 Hz, NH), 8.02 (4 H, m, 4 × NH), 8.19 (4 H, m, 4 × NH)

δ 0.82 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 1.22 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (17 H, m, Bu-t + Leu β-$H_2$ + Lys β,γ,δ-$H_2$ + 2 × $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.50 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.59-1.92 (3 H, m, Leu γ-H + Glu β-$H_2$), 2.04 (4 H, m, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.27 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76-3.18 (18 H, m, Sar-$H_3$ + Phe β-$H_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + 2 × $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.57 (3 H, s, $OCH_3$), 3.65 (4 H, m, 2 × Gly-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 3.92 (2 H, br, Sar-$H_2$), 4.20 (3H, m, 3 × α-H), 4.49 (1 H, m, 1 × α-H), 5.04 (2 H, s, Ar-$H_2$), 5.05 (2 H, s, Ar-$H_2$), 6.78 (1 H, t, $J$ = 6.7 Hz, NH), 7.22 (5 H, m, Ar), 7.36 (10 H, m, Ar), 7.78 (1 H, d, $J$ = 6.7 Hz, NH), 7.91 (1 H, d, $J$ = 7.8 Hz, NH), 8.02 (4 H, m, 4 × NH), 8.19 (4 H, m, 4 × NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C (COSY 90)

δ 0.82 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.1 Hz, Leu-$H_3$), 1.21 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (17 H, m, Bu-t + Leu β–$H_2$ + Lys β,γ,δ-$H_2$ + 2 × $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.49 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.59 (1 H, m, Leu γ-H), 1.76 (1 H, m, Glu β-$H_2$), 1.89 (1 H, m, Glu β-$H_2$), 2.02 (2 H, t, $J$ = 6.9 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.10 (2 H, t, $J$ = 5.2 Hz, Glu γ-$H_2$), 2.29 (2 H, t, $J$ = 6.9 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.78 (4 H, m, Phe β-$H_2$ + Sar-$H_3$), 3.02 (7 H, m, Phe β-$H_2$ + Lys ε-$H_2$ + 2 × $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.14 (4 H, br, $NCH_2CH_2N$), 3.57 (3 H, s, $OCH_3$), 3.69 (4 H, m, 2 × Gly-$H_2$), 3.81 (2 H, s, Sar-$H_2$), 3.89 (2 H, s, Sar-$H_2$), 4.18 (1 H, m, 1 × α-H), 4.22 (2 H, m, 2 × α-H), 4.52 (1 H, m, Phe α-H), 5.04 (2 H, s, Ar-$H_2$), 5.07 (2 H, s, Ar-$H_2$), 7.22 (5 H, m, Ar), 7.32 (10 H, m, Ar), 7.41 (1 H, br, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 7.46 (1 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 7.59 (1 H, d, $J$ = 8 Hz, NH (couples to signal at 4.18)), 7.68 (3 H, br, $N\underline{H}CH_2CH_2N\underline{H}$ + NH), 7.75 (1 H, m, Gly-NH), 7.82 (1 H, m, Phe-NH), 7.84 (1 H, m, NH (couples to signal at 4.22)), 7.87 (1 H, m, Gly-NH), 8.00 (1 H, m, NH (couples to signal at 4.22))

T.L.C. (C) Rf = 0.30

Acc. Mass 1441.7918 (M + H) ($C_{72}H_{107}N_{13}O_{18}$ requires 1441.7857)

**$N^{\varepsilon}$-(6-Aminohexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (260)**

Hydrogen chloride was passed through $N^{\varepsilon}$-(6-(1,1-dimethylethoxycarbonylamino)-hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (1.92 g, 1.32 mmol) in $CH_2Cl_2$ (20 ml) and MeOH (5 ml) for 40 min. The solvents and excess reagent were evaporated to give $N^{\varepsilon}$-(6-amino-hexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide hydrochloride (1.84g, quantitative).

This material was carried forward without characterisation.

**$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-tri-phenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (261)**

5-(4-(4-Nitrophenoxycarbonylamino)phenyl)-10,15,20-triphenyl-21*H*,23*H*-porphine (1.51 g, 1.94 mmol) was added to $N^{\varepsilon}$-(6-aminohexanoyl)-$N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)glutaminyl)glycyl)phenyl-alanyl)leucyl)glycyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.80 g, 1.29 mmol), N,N-dimethylethylamine (560 mg, 6.45 mmol), and DMAP (20 mg) in $CHCl_3$ (10 ml) and DMF (20 ml). The solution was stirred for 48 h. The solvents were evaporated. Chromatography ($CHCl_3$ / MeOH 20 : 1) gave $N^{\alpha}$-(N-(N-(N-(N-($N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-$N^{\delta}$-(6-methoxy-6-oxohexyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide hydrochloride (2.19 g, 85%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -2.95 (2 H, s, *21,23*H), 0.78 (3 H, d, $J$ = 5.9 Hz, Leu-$H_3$), 0.83 (3 H, d, $J$ = 5.9 Hz, Leu-$H_3$), 1.18 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.30 (6 H, m, Lys

β,γ,δ-$H_2$ + 2 × $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.46 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.55-1.95 (5 H, m, Leu γ-H + Leu β-$H_2$ + Glu β-$H_2$), 2.04 (4 H, m, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.21 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76-3.18 (18 H, m, Sar-$H_3$ + Phe β-$H_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + 2 × $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.52 (3 H, s, $OCH_3$), 3.59-3.78 (4 H, m, 2 × Gly-$H_2$), 3.79 (2 H, br, Sar-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 4.22 (3H, m, 3 × α-H), 4.51 (1 H, m, 1 × α-H), 4.97 (2 H, s, Ar-$H_2$), 4.99 (2 H, s, Ar-$H_2$), 6.35 (1 H, t, $J$ = 6 Hz, NH), 7.28 (15 H, 2 × Ar + Phe-Ar), 7.78 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.0-8.1 (8 H, m, disubstituted aromatic 2,6-$H_2$ + 6 × NH), 8.18 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 3 × NH), 8.75-8.95 (8 H, m, *2,3,7,8,12,13,17,18*-$H_8$)

δ -2.95 (2 H, s, *21,23*H), 0.78 (3 H, d, $J$ = 5.9 Hz, Leu-$H_3$), 0.83 (3 H, d, $J$ = 5.9 Hz, Leu-$H_3$), 1.18 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.30 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.46 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.55-1.95 (5 H, m, Leu γ-H + Leu β-$H_2$ + Glu β-$H_2$), 2.04 (4 H, m, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.21 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.76-3.18 (18 H, m, Sar-$H_3$ + Phe β-$H_2$ + Lys ε-$H_2$ + $NCH_2CH_2N$ + 2 × $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.52 (3 H, s, $OCH_3$), 3.59-3.78 (4 H, m, 2 × Gly-$H_2$), 3.79 (2 H, br, Sar-$H_2$), 3.84 (2 H, br, Sar-$H_2$), 4.22 (3H, m, 3 × α-H), 4.51 (1 H, m, 1 × α-H), 4.99 (2 H, s, Ar-$H_2$), 5.02 (2 H, s, Ar-$H_2$), 6.35 (1 H, t, $J$ = 6 Hz, NH), 7.28 (15 H, 2 × Ar + Phe-Ar), 7.78 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.0-8.1 (8 H, m, disubstituted aromatic 2,6-$H_2$ + 6 × NH), 8.18 (11 H, m, 3 × Ph 2,6-$H_2$ + disubstituted aromatic 3,5-$H_2$ + 3 × NH), 8.75-8.95 (8 H, m, *2,3,7,8,12,13,17,18*-$H_8$)

T.L.C. (C) Rf = 0.34

MS (FAB+) 1998 (M + H)

**$N^{\delta}$-(6-Hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (264)**

Hydrazine hydrate (0.4 ml, 7.19 mmol) was added to $N^{\delta}$-(6-methoxy-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (512 mg, 0.71 mmol) in MeOH (5 ml). The solution was heated at 40° C for 14 h. The solvent and excess reagent were evaporated to reveal $N^{\delta}$-(6-hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (512 mg, quantitative) as a cream solid. Mp 140-143°C

$^1$H NMR ($(CD_3)_2SO$)

δ 1.19 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.35 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.44 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.73 (1 H, m, Glu β-$H_2$), 1.84 (1 H, m, Glu β-$H_2$), 1.98 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.04 (2 H, m, Glu γ-$H_2$), 2.85 (2 H, s, Sar-$H_3$), 2.88 (2 H, s, Sar-$H_3$), 2.99 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.83 (2 H, s, Sar-$H_2$), 3.91 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Glu α-H), 5.04 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.36 (10 H, m, Ar), 7.78 (1 H, br, NH), 8.01 (1 H, br, NH), 8.04 (1 H, br, NH), 8.17 (1 H, m, NH), 8.92 (1 H, s, $NH_2N\underline{H}$)

δ 1.19 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.35 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.44 (2 H, m, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.73 (1 H, m, Glu β-$H_2$), 1.84 (1 H, m, Glu β-$H_2$), 1.98 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.04 (2 H, m, Glu γ-$H_2$), 2.84 (2 H, s, Sar-$H_3$), 2.88 (2 H, s, Sar-$H_3$), 2.99 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.12 (4 H, br, $NCH_2CH_2N$), 3.83 (2 H, s, Sar-$H_2$), 3.93 (2 H, s, Sar-$H_2$), 4.19 (1 H, m, Glu α-H), 5.03 (2 H, s, Ar-$CH_2$), 5.08 (2 H, s, Ar-$CH_2$), 7.36 (10 H, m, Ar), 7.78 (1 H, br, NH), 8.01 (1 H, br, NH), 8.04 (1 H, br, NH), 8.17 (1 H, m, NH), 8.92 (1 H, s, $NH_2N\underline{H}$)

T.L.C. (C) Rf = 0.18

MS (FAB+) 727 (M + H)

Acc. Mass (FAB+) 727.3768 (M + H) ($C_{35}H_{51}N_8O_9$ requires 727.3779).

**$N^{\delta}$-(6-Oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (270)**

4M Hydrogen chloride in dioxane (0.62 ml) was added to $N^{\delta}$-(6-hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (500 mg, 0.69 mmol) in DMF (2 ml). The solution was cooled to -20°C and *t*-butyl nitrite (0.074 ml) was added. The solution was stirred for 50 min and then cooled to -60°C. N,N-diisopropylethylamine (0.48 ml, 4 eq.) was added and the solution was allowed to warm to -30°C. 4-(10,15,20-Triphenyl-21*H*,23*H*-porphin-5-yl)-benzeneamine (1.28 g, 2.07 mmol) and DMAP (5 mg) in $CHCl_3$ (20 ml) were added to the solution. The solution was stirred at room temperature for 24 h. The solvents were

evaporated. Chromatography (hexane / EtOH 1:1) gave $N^{\delta}$-(6-oxo-6-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (332 mg, 37%) as a purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.75 (2 H, *21,23*-$H_2$), 0.89 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.25-1.65 (6 H, m, $NCH_2C\underline{H_2}CH_2C\underline{H_2}CH_2$ + Glu β-$H_2$), 1.9-2.4 (4 H, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.0-3.1 (8 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + 2 × Sar-$H_3$), 3.35 (4 H, br, $NCH_2CH_2N$), 3.85-4.05 (4 H, m, 2 × Sar-$H_2$), 4.42 (1 H, m, Glu α-H), 5.13 (4 H, br, 2 × Ar-$CH_2$), 7.25-7.33 (13 H, m, 3 × NH + 2 × Ar-$H_5$), 7.70-7.76 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.96 (2 H, m, 2 × NH), 8.12 (2 H, d, $J$ = 8.2 Hz, disubstituted aromatic 3,5-$H_2$), 8.17-8.23 (6 H, m, 3 × Ph 2,6-$H_2$), 8.85 (8 H, br, porphyrin *2,3,7,8,12,13,17,18*-$H_8$).

T.L.C. (C) Rf = 0.43

**$N^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)-ethylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (271)**

4M Hydrogen chloride in dioxane (1.86 ml) was added to a solution of $N^{\delta}$-(6-hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (1.50 g, 2.07 mmol) in DMF (3 ml). The solution was taken to -20°C and *t*-butyl nitrite (0.23 ml) was added. The solution was stirred for 3 h and then taken to -60°C. N,N-Dimethylethylamine (1.06 g, 8.27 mmol) was added and the solution warmed to -30°C. 5-(4-(Glycylamino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine trihydrochloride (2.30 g, 2.9 mmol) and N,N-dimethylethyl-amine (1.12 g, 8.7 mmol) were added and the solution warmed to room temperature. The solution was stirred for 24 h. The solvents were evaporated. Chromatography ($CHCl_3$ / MeOH 20 : 1) gave $N^{\delta}$-(6-oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-

yl)phenylamino)ethylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.93 g, 76%) as a deep purple glass.

$^1$H NMR ($CDCl_3$)

δ -2.75 (2 H, *21,23*-$H_2$), 0.89 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.25-1.65 (6 H, m, $NCH_2C\underline{H_2}CH_2C\underline{H_2}CH_2$ + Glu β-$H_2$), 1.9-2.4 (4 H, Glu γ-$H_2$ + $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 3.0-3.1 (8 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + 2 × Sar-$H_3$), 3.35 (4 H, br, $NCH_2CH_2N$), 3.85-4.05 (4 H, m, 2 × Sar-$H_2$), 4.15-4.25 (2 H, m, Gly-$H_2$), 4.42 (1 H, m, Glu α-H), 5.13 (4 H, br, 2 × Ar-$CH_2$), 7.25-7.33 (14 H, m, 4 × NH + 2 × Ar-$H_5$), 7.70-7.76 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.96 (2 H, m, 2 × NH), 8.12 (2 H, d, $J$ = 8.2 Hz, disubstituted aromatic 3,5-$H_2$), 8.17-8.23 (6 H, m, 3 × Ph 2,6-$H_2$), 8.84 (8 H, br, porphyrin *2,3,7,8,12,13,17,18*-$H_8$).

T.L.C. (C) Rf = 0.35

Acc. Mass (FAB+) 1381.6184 (M + H) ($C_{81}H_{81}N_{12}O_{10}$ requires 1381.6199).

**N-(N-(N-(N-(N$^{\delta}$-(6-Hydrazino-6-oxohexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (272)**

Hydrazine hydrate (0.52 ml, 9.3 mmol) was added to N-(N-(N-(N-(N$^{\delta}$-(6-methoxy-6-oxohexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonylsarcosylamino)ethyl)amide (1.00 g, 0.91 mmol) in MeOH (15 ml). The solution was heated at 45°C for 48 h. The solvent and excess reagent were evaporated to yield N-(N-(N-(N-(N$^{\delta}$-(6-hydrazino-6-oxohexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.00 g, quantitative) as a pale yellow glass.

$^1$H NMR ($(CD_3)_2SO$)

δ 0.81 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.05 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.22 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.48 (2 H, m, Leu β-$H_2$), 1.60 (1 H, m, Leu γ-H), 1.71-1.87 (2 H, m, Glu β-$H_2$), 1.97 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.06 (2 H, m, Glu γ-$H_2$), 2.77(1 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 2.96 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + 3.10 (4 H, br. $NCH_2CH_2N$), 3.52-3.69 (4 H, m, 2 × Gly α-H), 3.82 (2 H, br, Sar-$H_2$), 3.90 (2 H, br, Sar-$H_2$), 4.23 (2 H, m, Leu α-H + Glu α-H), 4.49 (1 H, m, Phe α-H), 4.98 (2 H, s, Ar-$CH_2$), 5.06 (2 H, s, Ar-$CH_2$), 7.28 (15 H, m, 3 × Ar), 7.76 (1 H, d, $J$ =4.9 Hz, NH), 7.84 (1 H, br, NH), 8.03 (2 H, br, 2 × NH), 8.07 (1 H, m, NH), 8.22 (3 H, m, 3 × NH) 8.91 (1 H, s, $N\underline{H}NH_2$)

δ 0.81 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.86 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.05 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.22 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.36 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.48 (2 H, m, Leu β-$H_2$), 1.60 (1 H, m, Leu γ-H), 1.71-1.87 (2 H, m, Glu β-$H_2$), 1.97 (2 H, t, $J$ = 7.3 Hz, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.06 (2 H, m, Glu γ-$H_2$), 2.77(1 H, m, Phe β-$H_2$), 2.82 (3 H, s, Sar-$H_3$), 2.85 (3 H, s, Sar-$H_3$), 2.96 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$ + 3.10 (4 H, br. $NCH_2CH_2N$), 3.52-3.69 (4 H, m, 2 × Gly α-H), 3.82 (2 H, br, Sar-$H_2$), 3.90 (2 H, br, Sar-$H_2$), 4.23 (2 H, m, Leu α-H + Glu α-H), 4.49 (1 H, m, Phe α-H), 5.01 (2 H, s, Ar-$CH_2$), 5.03 (2 H, s, Ar-$CH_2$), 7.28 (15 H, m, 3 × Ar), 7.76 (1 H, d, $J$ = 4.9 Hz, NH), 7.84 (1 H, br, NH), 8.03 (2 H, br, 2 × NH), 8.07 (1 H, m, NH), 8.22 (3 H, m, 3 × NH) 8.91 (1 H, s, $N\underline{H}NH_2$)

T.L.C. (C) Rf = 0.12

MS (FAB+) 1101 (M + H)

**N-(N-(N-(N-(N$^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)-phenylamino)ethylamino)hexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (274)**

4M Hydrogen chloride in dioxane (0.75 ml) was added to N-(N-(N-(N-(N$^{\delta}$-(6-hydrazino-6-oxohexyl)-N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (913 mg, 0.83 mmol) in DMF (3 ml). The solution was taken to -20°C and *t*-butyl nitrite (0.090 ml) was added. The solution was stirred for 3 h and then taken to -60°C. N,N-Di-methylethylamine (290 mg, 3.3 mmol) was added and the solution allowed to warm to

-30°C. 5-(4-(Glycylamino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine trihydrochloride (880 mg, 1.14 mmol), N,N-dimethylethylamine (396 g, 4.56 mmol) and DMAP (10 mg) in $CHCl_3$ (20 ml) were added. The solution was stirred for 48 h. The solvents were evaporated. Chromatography (a) ($CHCl_3$ / MeOH 20:1) and (b) $CHCl_3$ / MeOH 10:1) gave N-(N-(N-(N-($N^{\delta}$-(6-oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)ethylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)-sarcosylamino)ethyl)amide (1.21 g, 83%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -2.95 (2 H, s, *21,23*-$H_2$), 0.79 (3 H, d, $J$ = 6.3 Hz, Leu-$H_3$), 0.84 (3 H, d, $J$ = 6.3 Hz, Leu-$H_3$), 1.2-1.6 (6 H, m, $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 1.65-1.90 (5 H, m, Leu β-$H_2$ + Leu γ–H + Glu β-$H_2$), 2.06 (2 H, m, Glu γ-$H_2$), 2.20 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.75 (1 H, m, Phe β-$H_2$), 2.81 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 2.98 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$) 3.09 (4 H, br. $NCH_2CH_2N$), 3.55-3.78 (4 H, m, 2 × Gly α-H), 3.80 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.01 (1 H, m, α-H), 4.22(1 H, m, α-H), 4.50 (1 H, m, α-H), 4.98 (2 H, s, Ar-$CH_2$), 5.02 (2 H, s, Ar-$CH_2$), 7.28 (15 H, m, 3 × Ar), 7.82 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.02 (2 H, d, $J$ = 8.6 Hz, disubstituted aromatic-$H_2$), 8.14 (2 H, d, $J$ = 8.6 Hz, disubstituted aromatic-$H_2$), 8.18-8.22 (6 H, m, 3 × Ph 2,6-$H_2$), 8.80 (6 H, br, porphyrin *2,3,7,8,12,13,17,18*-$H_6$)

δ -2.95 (2 H, s, *21,23*-$H_2$), 0.79 (3 H, d, $J$ = 6.3 Hz, Leu-$H_3$), 0.84 (3 H, d, $J$ = 6.3 Hz, Leu-$H_3$), 1.2-1.6 (6 H, m, $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 1.65-1.90 (5 H, m, Leu β-$H_2$ + Leu γ–H + Glu β-$H_2$), 2.06 (2 H, m, Glu γ-$H_2$), 2.20 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.75 (1 H, m, Phe β-$H_2$), 2.83 (3 H, s, Sar-$H_3$), 2.89 (3 H, s, Sar-$H_3$), 2.98 (3 H, m, Phe β-$H_2$ + $NC\underline{H_2}CH_2CH_2CH_2CH_2$) 3.09 (4 H, br. $NCH_2CH_2N$), 3.55-3.78 (4 H, m, 2 × Gly α-H), 3.80 (2 H, br, Sar-$H_2$), 3.88 (2 H, br, Sar-$H_2$), 4.01 (1 H, m, α-H), 4.22(1 H, m, α-H), 4.50 (1 H, m, α-H), 5.02 (2 H, s, Ar-$CH_2$), 5.04 (2 H, s, Ar-$CH_2$), 7.28 (15 H, m, 3 × Ar), 7.82 (9 H, m, 3 × Ph 3,4,5-$H_3$), 8.02 (2 H, d, $J$ = 8.6 Hz, disubstituted aromatic-$H_2$), 8.14 (2 H, d, $J$ = 8.6 Hz, disubstituted aromatic-$H_2$), 8.18-8.22 (6 H, m, 3 × Ph 2,6-$H_2$), 8.80 (6 H, br, porphyrin *2,3,7,8,12,13,17,18*-$H_6$)

T.L.C. (C) Rf = 0.36

MS (FAB-) 1756 (M - H)

**$N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-Hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (275)**

Hydrazine hydrate (0.4 ml, 6.43 mmol) was added to $N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-methoxy-6-oxyhexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)phenylalanyl)-leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxy-carbonyl)sarcosylamino)ethyl)amide (890 mg, 0.63 mmol) in MeOH (10 ml). The solution was heated at 40°C for 24 h. Hydrazine hydrate (0.4 ml, 6.43 mmol) was added and heating continued at 55°C for 48 h. The solvent was evaporated to reveal $N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)-glycyl)phenylalanyl)leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (890 mg, quantitative) as a cream solid. Mp 185-190°C

$^1$H NMR ($(CD_3)_2SO$)

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.21 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.37 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.56-1.85 (3 H, m, Leu β-H + Leu γ-$H_2$), 1.86-2.15 (4 H, m, Glu β-$H_2$ + Glu γ-$H_2$), 1.98 (2 H, t, $J$ = 7.7 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.84 (3 H, s, Sar-$H_3$), 2.87 (3 H, s, Sar-$H_3$), 2.99 (5 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.45 (2 H, m, Gly-$H_2$), 3.71 (2 H, m, Gly-$H_2$), 3.83 (2 H, br, Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.26 (2 H, m, 2 × α-H), 4.53 (1 H, m, α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.01 (2 H, s, Ar-$CH_2$), 5.03 (2 H, s, Ar-$CH_2$), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.68 (1 H, m, NH), 7.77 (1 H, m, NH), 7.94 (5 H, m, 5 × NH), 8.18 (3 H, m, 3 × NH), 8.92 (1 H, s, $N\underline{H}NH_2$).

δ 0.82 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.87 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 1.21 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.37 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.56-1.85 (3 H, m, Leu β-H + Leu γ-$H_2$), 1.86-2.15 (4 H, m, Glu β-$H_2$ + Glu γ-$H_2$), 1.98 (2 H, t, $J$ = 7.7 Hz, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.78 (1 H, m, Phe β-$H_2$), 2.86 (3 H, s, Sar-$H_3$), 2.88 (3 H, s, Sar-$H_3$), 2.99 (5 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$ + Phe β-$H_2$), 3.11 (4 H, br, $NCH_2CH_2N$), 3.45 (2 H, m, Gly-$H_2$), 3.71 (2 H, m, Gly-$H_2$), 3.83 (2 H, br,

Sar-$H_2$), 3.91 (2 H, br, Sar-$H_2$), 4.15 (1 H, m, α-H), 4.26 (2 H, m, 2 × α-H), 4.53 (1 H, m, α-H), 4.77 (2 H, s, $CH_2CCl_3$), 5.03 (2 H, s, Ar-$CH_2$), 5.07 (2 H, s, Ar-$CH_2$), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.68 (1 H, m, NH), 7.77 (1 H, m, NH), 7.94 (5 H, m, 5 × NH), 8.18 (3 H, m, 3 × NH), 8.92 (1 H, s, N<u>H</u>$NH_2$).

T.L.C. (C) Rf = 0.20

**$N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)-phenylamino)ethylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-glutaminyl)glycyl)phenylalanyl)leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)-lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (277)**

4M Hydrogen chloride in dioxane (0.53 ml) was added to $N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-hydrazino-6-oxohexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutaminyl)glycyl)-phenylalanyl)leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (839 mg, 0.59 mmol) in DMF (2 ml). The solution was cooled to -20°C and *t*-butyl nitrite (0.064 ml) was added. The solution was stirred for 6h then cooled to -60°C. N,N-Dimethylethylamine (205 mg, 2.36 mmol) was added and the solution allowed to warm to -30°C. 5-(4-(Glycylamino)phenyl)-10,15,20-triphenyl-21*H*-23*H*-porphine trihydrochloride (0.63 g, 0.81 mmol), N,N-dimethyl-ethylamine (205 mg, 2.36 mmol) and DMAP (10 mg) in $CHCl_3$ (20 ml) were added and the solution stirred for 48 h. The solvents were evaporated. Chromatography ($CHCl_3$ / MeOH 20:1) gave $N^{\alpha}$-(N-(N-(N-($N^{\delta}$-(6-oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)ethylamino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)-sarcosyl)glutaminyl)glycyl)phenylalanyl)leucyl)glycine $N^{\varepsilon}$-(2,2,2-trichloroethoxy-carbonyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.00 g, 83%) as a purple glass.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ -2.94 (2 H, s, *21,23*-$H_2$), 0.83 (3 H, d, *J* = 7.3 Hz, Leu-$H_3$), 0.88 (3 H, br, Leu-$H_3$), 1.18 (6 H, m, Lys β,γ,δ-$H_2$ + 2 × $NCH_2CH_2C\underline{H}_2CH_2CH_2$), 1.37 (6 H, m, Lys β,γ,δ-$H_2$ +

$NCH_2C\underline{H}_2CH_2CH_2CH_2$), 1.49 (4 H, m, Lys β,γ,δ-$H_2$ + $NCH_2CH_2CH_2C\underline{H}_2CH_2$), 1.50-1.90 (7 H, m, Leu β-$H_2$ + Leu γ-H + Glu β-$H_2$ + Glu γ-$H_2$) 2.05 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H}_2$), 2.76-2.91 (8 H, m, Phe β-$H_2$ + 2 × Sar-$H_3$), 3.08 (4 H, m, $NC\underline{H}_2CH_2CH_2CH_2CH_2$ + Lys ε-$H_2$), 3.10 (4 H, br, $NCH_2CH_2N$), 3.69 (4 H, m, 2 × Gly-$H_2$), 3.81 (2 H, br, Sar-$H_2$), 3.89 (2 H, br, Sar-$H_2$), 4.18 (3 H, m, 3 × α-H), 4.50 (1 H, m, α-H), 4.74 (2 H, s, $CH_2CCl_3$), 5.01 (2 H, br, Ar-$CH_2$), 5.05 (2 H, br, Ar-$CH_2$), 7.30 (15 H, m, 2 × Ar + Phe-Ar), 7.70-7.76 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic 2,6-$H_2$), 7.96 (2 H, m, 2 × NH), 8.12 (2 H, d, $J$ = 8.2 Hz, disubstituted aromatic 3,5-$H_2$), 8.17-8.23 (12 H, m, 6 × NH + 3 × Ph 2,6-$H_2$), 8.84 (12 H, br, 4 × NH + porphyrin *2,3,7,8,12,13,17,18*-$H_8$).

T.L.C. (C) Rf = 0.33

MS (FAB+) 2059 (M + H)

**N-(Phenylmethoxycarbonyl)sarcosine N-(1-methylethyl)amide (278)**

N-(1-methylethyl)amine (1.82g, 30.8 mmol) was added dropwise to a stirred solution of N-(phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (4.0 g, 10.3 mmol) in $CH_2Cl_2$ (20 ml). After 2 h, the solution was washed with water, twice with 10% aq. $H_2SO_4$ and twice with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated to give N-(phenylmethoxycarbonyl)sarcosine N-(1-methylethyl)amide (2.5 g, 92%) as an off-white solid. Mp 91 - 93°C.

$^1$H NMR ($CDCl_3$)

δ 1.10 (6 H, br, $(C\underline{H}_3)_2CH$), 3.01 (3 H, s, Sar-$H_3$), 3.89 (2 H, s, Sar-$H_2$), 4.05 (1 H, m, $(CH_3)_2C\underline{H}$), 5.16 (2 H, s, Ar-$CH_2$), 5.7 (1 H, m, NH), 7.36 (5 H, br, Ar)

T.L.C. (C) Rf = 0.61

**Sarcosine N-(1-methylethyl)amide hydrobromide (279)**

N-(Phenylmethoxycarbonyl)sarcosine N-(1-methylethyl)amide (1.0 g, 3.8 mmol) was dissolved in a 30% solution of HBr in acetic acid (5.1 g, 18.9 mmol). After 1 h, the

reaction was quenched by addition of dry $Et_2O$ (75 ml). The mixture was allowed to stand at 4°C for 1 h. The precipitate was then collected by filtration to give sarcosine N-(1-methylethyl)amide hydrobromide (680 mg, 85%) as a buff solid. Mp 148 - 150°C.

$^1H$ NMR ($CDCl_3$)

δ 1.17 (6 H, d, $J$ = 6.6 Hz, $(C\underline{H}_3)_2CH$), 2.65 (3 H, t, $J$ = 5.3 Hz, Sar-$H_3$), 3.70 (2 H, t, $J$ = 5.7 Hz, Sar-$H_2$), 4.01 (1 H, m, $(CH_3)_2C\underline{H}$), 8.30 (1 H, d, $J$ = 7.1 Hz), 9.05 (2 H, br, $NH_2^+$)

T.L.C. (C) Rf = 0.18

**N-(2-Hydroxy-3-phenoxypropyl)sarcosine N-(1-methylethyl)amide (280)**

2-(Phenoxymethyl)oxirane (284 mg, 1.9 mmol) was added to a solution of sarcosine N-(1-methylethyl)amide hydrobromide (400 mg, 1.9 mmol) and N,N-diisopropylethylamine (269 mg, 2.1 mmol) in ethanol and the resulting solution was stirred for 3 d. The solvent was evaporated and the residue was dissolved in $CH_2Cl_2$. The solution was washed with water and was then dried. The solvent was evaporated to give N-(2-hydroxy-3-phenoxy-propyl)sarcosine N-(1-methylethyl)amide (430 mg, 90%) as a yellow oil.

$^1H$ NMR ($CDCl_3$)

δ 1.16 (6 H, d, $J$ = 6.7 Hz, $(C\underline{H}_3)_2CH$), 2.40 (3 H, s, Sar-$H_3$), 2.55 (1 H, dd, $J$ = 12.8, 3.3 Hz, $NC\underline{H}_2CH$), 2.67 (1 H, dd, $J$ = 12.8, 9.2 Hz, $NC\underline{H}_2CH$), 3.89 (1 H, dd, $J$ = 9.5, 6.4 Hz, $OC\underline{H}_2CH$), 3.99 (1 H, dd, $J$ = 9.5, 4.0 Hz, $OC\underline{H}_2CH$), 4.07 (1 H, m, $CH_2C\underline{H}CH_2$), 4.12 (1 H, m, $(CH_3)_2C\underline{H}$), 6.89 (2 H, t, $J$ = 7.9 Hz, Ar-2,6), 6.98 (1 H, t, $J$ = 7.3 Hz, Ar-4), 7.29 (2 H, m, Ar-3,5)

T.L.C. (C) Rf = 0.55

MS (CI) 281 (M + H).

**N-(Phenylmethoxycarbonyl)sarcosine N-(12-((N-phenylmethoxycarbonyl)sarcosyl-amino)-4,9-dioxa-1,12-dodecanyl)amide (282)**

N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (10.56 g, 26.25 mmol) was added to 4,9-dioxadodecane-1,12-diamine (2.14 g, 10.5 mmol), N,N-diisopropyl-ethylamine (2.98 g, 23.1 mmol) and DMAP (20 mg) in $CH_2Cl_2$ (40 ml). The solution was stirred for 4 d. The solution was washed with 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ then $CH_2Cl_2$ / MeOH 40:1) yielded N-(phenylmethoxycarbonyl)sarcosine N-(12-((N-phenyl-methoxycarbonyl)sarcosylamino)-4,9-dioxa-1,12-dodecanyl)amide (5.00 g, 78%) as a cream wax.

$^1$H NMR ($CDCl_3$)

δ 1.56 (4 H, s, $OCH_2C\underline{H_2}C\underline{H_2}CH_2O$), 2.99 (3 H, s, Sar-$H_3$), 3.37 (8 H, m, 2 × $CH_2C\underline{H_2}OC\underline{H_2}$), 3.44 (4 H, br, 2 × $C\underline{H_2}CH_2OCH_2$), 5.14 (4 H, s, Sar-$H_2$), 6.54 (2 H , br, 2 × NH), 7.34 (10 H, br, Ar)

T.L.C. (C) Rf = 0.56

MS (FAB+) 615 (M + H)

**N-(Phenylmethoxycarbonyl)sarcosine N-(9-((N-phenylmethoxycarbonyl)sarcosyl-amino)nonyl)amide (286)**

N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (6.33 g, 15.75 mmol) was added to nonane-1,9-diamine (1.00g, 6.30 mmol), N,N-diisopropylethylamine (814 mg, 13.86 mmol) and DMAP (10 mg) in $CHCl_2$ (50 ml). The solution was stirred for 7 d. The solution was washed with 10% aq. $H_2SO_4$ and with 10% aq. $Na_2CO_3$. The solution was dried and the solvent was evaporated. Chromatography ($CH_2Cl_2$ / MeOH 40 : 1) gave N-(phenylmethoxycarbonyl)sarcosine N-(9-((N-phenylmethoxycarbonyl)-sarcosylamino)nonyl)amide (3.25 g, 91%) as a white solid. Mp 80-83°C.

$^1$H NMR ($CDCl_3$)

δ 1.25 (10 H, s, $NCH_2CH_2(C\underline{H_2})_5CH_2CH_2N$), 1.43 (4 H, br, $NCH_2C\underline{H_2}(CH_2)_5$-$C\underline{H_2}CH_2N$), 3.01 (6 H, s, 2 × Sar-$H_3$), 3.22 (4 H, m, $NC\underline{H_2}CH_2(CH_2)_5CH_2C\underline{H_2}N$), 3.91 (4 H, s, 2 × Sar-$H_2$), 5.16 (3 H, s, Ar-$CH_2$), 5.85 (1 H, br, NH), 6.10 (1 H, br, NH), 7.35 (10 H, s, Ar)

T.L.C. (C) Rf = 0.55

**Sarcosine N-(9-(sarcosylamino)nonyl)amide (287)**

10% Palladium on charcoal (450 mg) was added to a solution of N-(phenylmethoxy-carbonyl)sarcosine N-(9-((N-phenylmethoxycarbonyl)sarcosylamino)nonyl)amide (2.26 g, 3.98 mmol) in MeOH (30 ml). The mixture was treated with hydrogen for 2 d and then filtered through Celite®. The solvent and MeOH washings were evaporated to give sarcosine N-(9-(sarcosylamino)nonyl)amide (1.19 g, 99%) as a white wax.

$^1$H NMR ($CDCl_3$)

δ 1.23 (10 H, s, $NCH_2CH_2(C\underline{H_2})_5CH_2CH_2N$), 1.38 (4 H, br, $NCH_2C\underline{H_2}(CH_2)_5$-$C\underline{H_2}CH_2N$), 2.22 (6 H, s, 2 × Sar-$H_3$), 2.98 (4 H, s, 2 × Sar-$H_2$), 3.05 (4 H, q, $J$ = 6.2 Hz, $NC\underline{H_2}CH_2(CH_2)_5CH_2C\underline{H_2}N$), 7.74 (2 H, br, 2 × NH)

T.L.C. (C) Rf = 0.14

**1,14-Bis(oxiranylmethoxy)-3,6,9,12-tetraoxatetradecane (289)**

Tetrabutylammonium hydroxide (40% w/v in water, 162 mg, 0.25 mmol) was added to a mixture of chloromethyloxirane (6.75 g, 75 mmol), NaOH (3.0 g, 7 mmol) and water (0.3 ml). Penta(ethylene glycol) (3,6,9,12-tetraoxatetradecane-1,14-diol) (2.98 g, 12.5 mmol) was added dropwise to this stirred solution. The solution was heated at 40°C for 40 min. The suspension was then filtered and the organic layer was diluted with $CH_2Cl_2$.The solution was dried and the solvents were evaporated to give 1,14-bis(oxiranylmethoxy)-3,6,9,12-tetraoxatetradecane (4.04 g, quantitative) as a colourless liquid.

$^1$H NMR ($CDCl_3$)

δ 2.62 (2 H, dd, $J$ = 5.1, 2.8 Hz, 2 × oxirane 3-H), 2.80 (2 H, t, $J$ = 4.2 Hz, 2 × oxirane 3-H), 3.17 (2 H, m, 2 × oxirane 2-H), 3.42 (2 H, dd, $J$ = 11.5, 5.7 Hz, 2 × $OCH_2$-oxirane), 3.66 (20 H, 5 × $OCH_2CH_2O$), 3.81 (2 H, dd, $J$ = 11.5, 2.9 Hz, 2 × $OCH_2$-oxirane)

**PEG-400 bis(oxiranylmethyl ether) (291)**

**Method 1**

PEG-400 (5.00 g, 12.5 mmol) was added dropwise to a stirred solution of chloromethyl-oxirane (6.75 g, 75 mmol), NaOH (3.00 g, 7.5 mmol), tetrabutylammonium hydroxide (40% in water, 0.162 g) and water (0.3 ml). The suspension was heated at 65°C for 2 h. The cooled suspension was filtered and the solids were washed with $CH_2Cl_2$. The combined filtrate and washings were dried and the solvent was evaporated to afford PEG-400 bis(oxiranylmethyl ether) (4.24 g, 68%)

$^1$H NMR ($CDCl_3$)

δ 2.59 (2 H, dd, $J$ = 5.1, 2.4 Hz, 2 × oxirane 3-H), 2.78 (2 H, t, $J$ = 5.0 Hz, 2 × oxirane 3-H), 3.15 (2 H, m, 2 × oxirane 2-H), 3.40 (2 H, dd, $J$ = 11.7, 5.8 Hz, 2 × $OCH_2$-oxirane), 3.66 (35 H, 9 × $OCH_2CH_2O$), 3.78 (2 H, dd, $J$ = 11.5, 2.9 Hz, 2 × $OCH_2$-oxirane)

**PEG-400 bis(oxiranylmethyl ether) (291)**

**Method 2**

PEG-400 (5.00 g, 12.5 mmol) was added dropwise to a stirred solution of chloromethyl-oxirane (6.75 g, 75 mmol), NaOH (3.00 g, 7.5 mmol) and water (0.3 ml). The suspension was heated at 65°C for 2 h. The cooled suspension was filtered and the solids were washed with $CH_2Cl_2$. The combined filtrate and washings were dried and the solvent was evaporated to afford PEG-400 bis(oxiranylmethyl ether) (4.49 g, 71%). This material was spectroscopically identical to the material prepared by Method 1.

**Macrocycle derived from sarcosine N-(9-(sarcosylamino)nonyl)amide and PEG-400 bis(oxiranylmethyl ether) (292)**

Sarcosine N-(9-(sarcosylamino)nonyl)amide (500 mg, 1.66 mmol) and PEG-400 bis(oxiranylmethyl ether) (806 mg, 1.66 mmol) were heated at reflux in ethanol (10 ml) for 15 h. The solution was allowed to cool and the solvent was evaporated to give the macrocycle (1.30 g, 99%).

$^1$H NMR ($CDCl_3$)

δ 1.29 (10 H, s, $NCH_2CH_2(C\underline{H}_2)_5CH_2CH_2N$), 1.49 (4 H, br, $NCH_2C\underline{H}_2(CH_2)_5$-$C\underline{H}_2CH_2N$), 2.34 (6 H, s, 2 × Sar-$H_3$), 2.42 (2 H, dd, J = 13, 4 Hz, 2 × $NC\underline{H}_2CHOH$), 2.50 (2 H, dd, J = 13, 8 Hz, 2 × $NC\underline{H}_2CHOH$), 3.05 (2 H, d, J = 16 Hz, Sar-$H_2$), 3.10 (2 H, d, J = 16 Hz, Sar-$H_2$), 3.23 (4 H, q, *J* = 7 Hz, $NC\underline{H}_2CH_2(CH_2)_5CH_2C\underline{H}_2N$), 3.35-3.55 (4 H, m, 2 × $OC\underline{H}_2CHOH$), 3.62 (35 H, br, 9 × $OCH_2CH_2O$), 3.91 (2 H, m, 2 × $C\underline{H}OH$), 7.45 (2 H, br, NH)

MS (electrospray +) 915 (M + H, $(CH_2CH_2O)_{11}$), 871 (M + H, $(CH_2CH_2O)_{10}$), 827 (M + H, $(CH_2CH_2O)_9$), 783 (M + H, $(CH_2CH_2O)_8$), 739 (M + H, $(CH_2CH_2O)_7$), 695 (M + H, $(CH_2CH_2O)_6$), 651 (M + H, $(CH_2CH_2O)_5$)

**PEG-1500 bis(oxiranylmethyl ether) (294)**

PEG-1500 (20.0 g, 13.3 mmol) was added dropwise to a stirred solution of chloromethyloxirane (14.37 g, 160 mmol), NaOH (3.20 g, 8.0 mmol) and water (0.31 ml). The suspension was heated at 65°C for 3 h. The cooled suspension was filtered and the solids were washed with $CH_2Cl_2$. The combined filtrate and washings were dried and the solvent was evaporated to afford PEG-1500 bis(oxiranylmethyl ether) (16.33 g, 76%).

$^1$H NMR ($(CD_3)_2SO$)

δ 2.52 (2 H, dd, *J* = 5.1, 2.7 Hz, 2 × oxirane 3-H), 2.71 (2 H, t, *J* = 5.0 Hz, 2 × oxirane 3-H), 3.08 (2 H, m, 2 × oxirane 2-H), 3.24 (2 H, dd, *J* = 11.5, 5.1 Hz, 2 × $OC\underline{H}_2$-oxirane), 3.66 (135 H, 34 × $OCH_2CH_2O$), 3.69 (2 H, dd, *J* = 11.5, 2.6 Hz, 2 × $OC\underline{H}_2$-oxirane).

**Gel Permeation Chromatography : Compound 292**

Analysis performed by Dr G. Price, School of Chemistry, University of Bath.

**Parameters**

Bruker LC21/41 GPC System

THF eluent 1 ml $min^{-1}$

'PL Gel' (Polymer Laboratories Ltd) 60 cm mixed bed column

75 µl of 0.01% solution injection volume

SUMMARY REPORT  Date : 30/08/94  Time : 08:03

| | | | | | |
|---|---|---|---|---|---|
| Chromatogram | : PH-C19 | | Sample Id. : | | |
| Baseline Mode | : Trapezoidal | | | | |
| Baseline drawn from | : 15.50 | to | 19.95 | (min) | |
| Integration from | : 15.58 | to | 19.93 | (min) | |
| Total Area | : 8.0522E+05 | | Nr. data points : | | 522 |

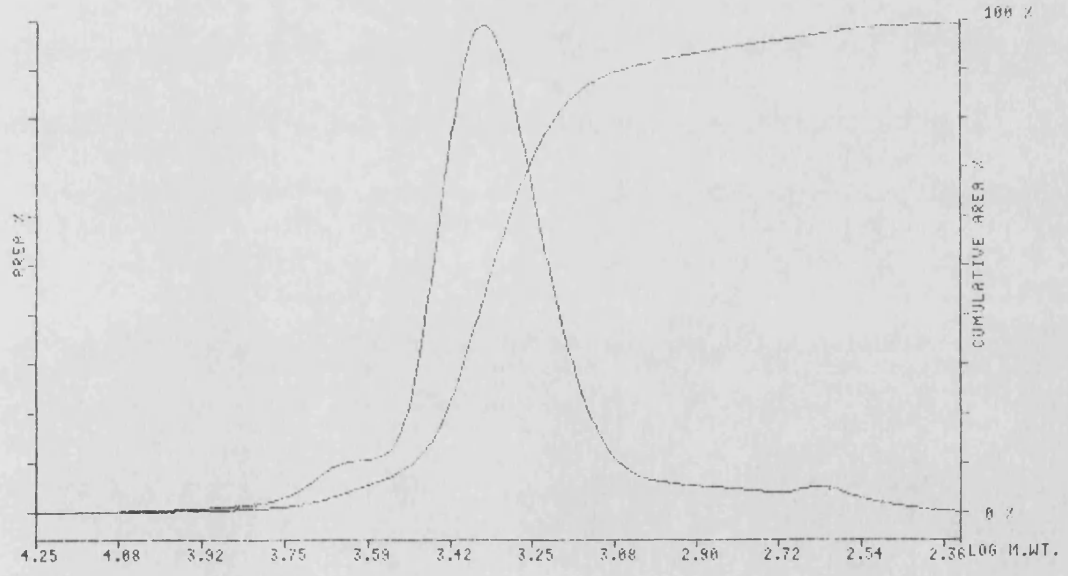
Average Molecular Weight

| | | |
|---|---|---|
| Number | (Mn) : | 1.7410E+03 |
| Weight | (Mw) : | 2.2601E+03 |
| Z- | (Mz) : | 2.7636E+03 |
| Z+1 | (Mz+1) : | 3.6049E+03 |
| Polydispersity | (D) : | 1.298 |

Results calculated using  Calibration Mode : Narrow Standard

| | | | |
|---|---|---|---|
| Data handling file | : MAIN | Mark-Houwink Constants : | |
| Calibration file | : 8-94 | K = 1.000000 | a = 0.00 |
| Fit Type | : Cubic | Ret. of Ref. Peak : | 0.00 |
| Coefficients : | | Ret. Window Peak : | 0.00 |

K0 : 15.0758  K1 : -1.24296  K2 : .0528998  K3 : -1.13624E-03




c

**N-(N-(N-(N-Sarcosylglycyl)phenylalanyl)leucyl)glycine N-(2-(N-sarcosylamino)-ethyl)amide (295)**

N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (500 mg, 0.59 mmol) was heated to 60°C in MeOH (30 ml). Palladium on charcoal (67 mg) was added to the stirred solution and the reaction mixture treated with hydrogen. After 30 h, the suspension was filtered and the solvent was evaporated from the filtrate to give N-(N-(N-(N-sarcosylglycyl)phenylalanyl)leucyl)glycine N-(2-(N-sarcosylamino)ethyl)amide (332 mg, 98%) as a colourless oil.

$^1$H NMR ($(CD_3)_2SO$) 20°C

δ 0.93 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 0.99 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.55 (3 H, m, Leu γ-H + Leu β-$H_2$), 2.97 (1 H, m, Phe β-$H_2$), 3.11 (1 H, m, Phe β-$H_2$), 3.16 (6 H, s, 2 × Sar-$H_3$), 3.27 (4 H, br, $NCH_2CH_2N$), 3.48 (4 H, br, 2 × Sar-$H_2$), 3.68-3.85 (4 H, m, 2 × Gly-$H_2$), 4.28 (1 H, m, α-H), 4.62 (1 H, br, α-H), 7.34 (10 H, m, Ar), 7.86 (1 H, m, NH), 8.03 (1 H, m, NH), 8.16 (2 H, m, NH), 8.23 (1 H, m, NH), 8.34 (1 H, m, NH)

$^1$H NMR ($(CD_3)_2SO$) 80°C

δ 0.85 (3 H, d, $J$ = 6.4 Hz, Leu-$H_3$), 0.89 (3 H, d, $J$ = 6.2 Hz, Leu-$H_3$), 1.43 (1 H, m, Leu γ-H), 1.53 (2 H, m, Leu β-$H_2$), 2.86 (2 H, m, Phe β-$H_2$), 3.05 (3 H, s, Sar-$H_3$), 3.08 (3 H, s, Sar-$H_3$), 3.19 (4 H, br, $NCH_2CH_2N$), 3.57-3.82 (8 H, m, 2 × Sar-$H_2$ + 2 × Gly-$H_2$), 4.25 (1 H, m, α-H), 4.55 (1 H, br, α-H), 7.22 (10 H, m, Ar), 7.55 (1 H, br, NH), 7.69 (2 H, br, NH), 7.87 (3 H, br, NH).

T.L.C. (C) Rf = 0.05

**Polymerisation of N-(N-(N-(N-sarcosyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-sarcosylamino)ethyl)amide and PEG-1500 bis(oxiranylmethyl ether) (296)**

N-(N-(N-(N-sarcosyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-sarcosylamino)ethyl)-amide (120 mg, 0.21 mmol), and PEG-1500 bis(oxiranylmethyl ether) (312 mg, 0.21 mmol) were heated at reflux in ethanol (5 ml) for 48 h. The solution was allowed to

cool and the solvent was evaporated to give a colourless gum. Samples of the crude product were taken for GPC analysis.

**$N^{\alpha}$-Sarcosyl-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)-ureido)hexanoyl)lysine N-(2-(N-sarcosylamino)ethyl)amide tetrahydrobromide (297)**

$N^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide (150 mg, 0.11 mmol) was dissolved in acetic acid (2 ml). HBr in acetic acid (30%) (1 ml) was added and the solution was stirred for 45 min. The salt was precipitated by addition of ether (20 ml). The solid was washed four times with ether and was then collected by filtration. The solid was dried under reduced pressure in the presence of NaOH to give $N^{\alpha}$-sarcosyl-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-sarcosylamino)ethyl)amide tetra-hydrobromide (151 mg, 97%) as a dark green glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -0.60 (2 H, br, *21,22,22,24*-$H_2$), -0.38 (2 H, br, *21,22,22,24*-$H_2$), 1.2-1.8 (12 H, m, Lys β,γ,δ-$H_2$ + $NCH_2C\underline{H_2}C\underline{H_2}C\underline{H_2}CH_2$), 2.12 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.56 (6 H, m, 2 × Sar-$H_3$), 3.05 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$) 3.19 (6 H, Lys ε-$H_2$ + $NCH_2CH_2N$), 3.70 (2 H, t, $J$ = 5.5 Hz, Sar-$H_2$), 3.78 (2 H, m, Sar-$H_2$), 4.24 (1 H, m, Lys α-H), 7.80 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic), 8.05-8.30 (15 H, br, disubstituted aromatic 2,3,5,6-$H_4$ + 3 × Ph 2,6-$H_2$, + 5 × NH), 8.59 (1 H, br, NH), 8.6-8.9 (12 H, br, *2,3,7,8,12,13,17,18*-$H_4$ + 2 × $NH_2^+$)

UV (MeOH + N,N-diisopropylethylamine) λ 406 (Soret), 512, 549, 590, 645

**Polymerisation of $N^{\alpha}$-(N-sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)hexanoyl)lysine N-(2-(N-sarcosylamino)ethyl)amide and PEG-1500 bis(oxiranylmethyl ether) (298)**

$N^{\alpha}$-(N-sarcosyl)-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)-hexanoyl)lysine N-(2-(N-sarcosylamino)ethyl)amide tetrahydrobromide (100 mg, 0.08 mmol), $K_2CO_3$ (0.35 mmol) PEG-1500 bis(oxiranylmethyl ether) (105 mg, 0.07 mmol) were heated at reflux in ethanol (5 ml) for 48 h. The suspension was allowed to cool and was then filtered. The solvent was evaporated from the filtrate and washings to give a purple gum. Samples of the crude product were taken for GPC analysis.

$^1$H NMR ($CDCl_3$)

δ 1.3-1.7 (br), 2.2-2.5 (br), 3.1-4.0 (br), 7.6-7.8 (br), 7.9 (br), 8.0-8.3 (br), 8.7-9.0 (br).

***S*-Chloro-(5-(4-(N'-(6-oxo-6-(1-oxo-2-(sarcosylamino)-1-(2-(sarcosylamino)ethyl-amino)hexylamino)hexyl)ureido)phenyl)-10,15,20-triphenylporphinato)manganese (III) (299)**

$N^{\alpha}$-Sarcosyl-$N^{\varepsilon}$-(6-(N'-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenyl)ureido)-hexanoyl)lysine N-(2-(N-sarcosylamino)ethyl)amide tetrahydrobromide (100 mg, 0.08 mmol) was dissolved in an excess of conc. aq. HCl to form the hydrochloride salt. The solvent and excess reagent were evaporated and the residue was dissolved in DMF (10 ml). Manganese (II) chloride (10 mg, 0.08 mmol) was added and the suspension was heated at reflux for 24 h. A further portion of manganese (II) chloride was added (10 mg, 0.08 mmol) and heating continued. After a further 24 h, the suspension was filtered and the solvent was evaporated. The residue was dissolved in $CHCl_3$ . The solution was washed with water and with aq. $Na_2CO_3$ and was then dried. The solvent was evaporated to give *S*-chloro-(5-(4-(N'-(6-oxo-6-(1-oxo-2-(sarcosylamino)-1-(2-(sarcosylamino)-

ethylamino)hexylamino)hexyl)ureido)phenyl)-10,15,20-triphenylporphinato)manganese (III) (51 mg, 57%) as a dark green-brown gum.

UV (MeOH) λ 467 (Soret), 564, 600

**$N^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)-ethylamino)hexyl)-$N^{\alpha}$-(N-sarcosyl)glutamine N-(2-(N-sarcosylamino)ethyl)amide tetrahydrobromide (300)**

$N^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)ethyl-amino)hexyl)-$N^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)glutamine N-(2-(N-(phenyl-methoxycarbonyl)sarcosylamino)ethyl)amide (300 mg, 0.22 mmol) was dissolved in acetic acid (4 ml) and 30% HBr in acetic acid (2 ml). The solution was stirred for 1 h. Ether (40 ml) was added and the salt precipitated. The salt was washed four times with ether (40 ml), collected by filtration and then dried under reduced pressure in the presence of NaOH to give $N^{\delta}$-(6-oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)ethylamino)hexyl)-$N^{\alpha}$-(N-sarcosyl)glutamine N-(2-(N-sarcosylamino)ethyl)amide tetrahydrobromide (260 mg, 94%) as a dark green glass.

$^1$H NMR ($(CD_3)_2SO$)

δ -0.30 (2 H, br, *21,22,23,24*-$H_2$), -0.26 (2 H, br, *21,22,23,24*-$H_2$), 1.34 (2 H, m, $NCH_2CH_2C\underline{H_2}CH_2CH_2$), 1.45 (2 H, m, $NCH_2C\underline{H_2}CH_2CH_2CH_2$), 1.58 (2 H, $NCH_2CH_2CH_2C\underline{H_2}CH_2$), 1.70-1.95 (2 H, m, Glu β-$H_2$), 2.12 (2 H, m, Glu γ-$H_2$), 2.25 (2 H, m, $NCH_2CH_2CH_2CH_2C\underline{H_2}$), 2.57 (6 H, t, $J$ = 5.3 Hz, 2 × Sar-$H_3$), 3.06 (2 H, m, $NC\underline{H_2}CH_2CH_2CH_2CH_2$), 3.18 (4 H, br, $NCH_2CH_2N$), 3.7-4.02 (6 H, m, 2 × Sar-$H_2$ + Gly-$H_2$), 4.24 (1 H, m, Glu α-H), 7.82 (11 H, m, 3 × Ph 3,4,5-$H_3$ + disubstituted aromatic), 8.00-8.35 (15 H, br, disubstituted aromatic 2,3,5,6-$H_4$ + 3 × Ph 2,6-$H_2$, + 5 × NH), 8.51 (1 H, br, NH), 8.6-8.9 (12 H, br, *2,3,7,8,12,13,17,18*-$H_4$ + 2 × $NH_2^+$)

UV (MeOH + N,N-diisopropylethylamine) λ 404 (Soret), 509, 546, 588, 646

**_S_-Chloro-(5-(4-((N-(6-(1-oxo-2-(sarcosylamino)-1-(2-(sarcosylamino)ethylamino)-pentanoylamino)hexanoyl)glycyl)amino)phenyl)-10,15,20-triphenylporphinato)-manganese (III) (301)**

$N^{\delta}$-(6-Oxo-6-(2-oxo-2-(4-(10,15,20-triphenyl-21*H*,23*H*-porphin-5-yl)phenylamino)ethyl-amino)hexyl)-$N^{\alpha}$-(N-sarcosyl)glutamine N-(2-(N-sarcosylamino)ethyl)amide tetrahydro-bromide (277 mg, 0.22 mmol) and manganese (II) chloride (275 mg, 2.2 mmol) were heated at reflux in DMF for 2 h. The suspension was cooled and then filtered. The solvent was evaporated from the filtrate and the residue redissolved in $CHCl_3$. The solution was washed with $Na_2CO_3$ and then dried. The solvent was evaporated to give *S*-chloro-(5-(4-((N-(6-(1-oxo-2-(sarcosylamino)-1-(2-(sarcosylamino)ethylamino)pentanoylamino)-hexanoyl)glycyl)amino)phenyl)-10,15,20-triphenylporphinato)manganese (III) (190 mg, 72%) as an dark green-brown glass.

UV (MeOH) λ 467 (Soret), 564, 598

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# PUBLISHED WORK

**S. E. Matthews, C. W. Pouton and M. D. Threadgill**

Monofunctional Electrophilic and Nucleophilic Derivatives of *meso*-Tetraphenylporphyrin for Attachment to Peptides

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# Monofunctional Electrophilic and Nucleophilic Derivatives of *meso*-Tetraphenylporphyrin for Attachment to Peptides

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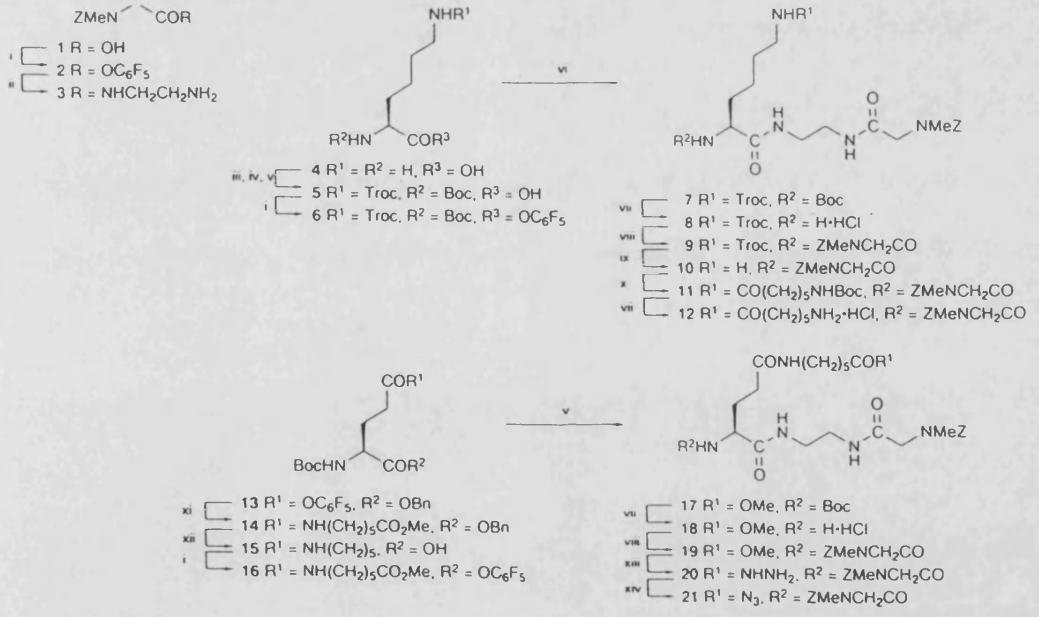
4-Nitrophenyl *N*-[4-(10,15,20-triphenylporphyrin-5-yl)phenyl]carbamate and 5-[4-(*N*-glycylamino)phenyl]-10,15,20-triphenylporphyrin have been synthesised from a readily prepared monofunctionalised porphyrin; they couple efficiently with the side-chains of extended lysyl and glutamyl peptide derivatives, respectively.

Porphyrins alone or linked to polymers and other targetting moieties have important roles in diagnosis and therapy of cancer. For example, the so-called 'haematoporphyrin derivative' and other porphyrins act as photosensitisers for conversion of triplet oxygen to singlet oxygen in photodynamic therapy[1] and porphyrins are known to accumulate selectively in some types of tumour tissue,[2] leading to prospects of their use as targetting groups. Porphyrinatomanganese complexes are used as contrast-enhancing agents in magnetic resonance imaging (MRI), owing to their high molar relaxivities in aqueous solution.[3] Attachment of metalloporphyrin and other agents to a soluble polymer enhances molar relaxivity and thus effectiveness as a MRI contrast agent.[4]

Significant difficulty in preparing mono-functionalised porphyrins in a controlled manner is caused by the fact that the most readily available naturally-occurring porphyrins, such as protoporphyrin IX and mesoporphyrin II, carry more than one identical electrophilic or nucleophilic functional group. The classical Adler–Rothemund[5] procedure for synthesis of *meso*-tetraarylporphyrins from arylaldehydes and pyrrole proceeds in < 20% yield and mono-Ar-functionalised porphyrins are only obtained in very low yields by separation of statistical mixtures of porphyrins formed from mixtures of aldehydes,[6] whereas the recently-reported Heck coupling[7] needs several synthetic steps to prepare the starting 5,15-diphenyl-10-iodoporphyrin. To obviate these problems of synthesis of mono-Ar-substituted tetraphenylporphyrins, Kruper *et al.*[8] developed an efficient mononitration and subsequent reduction of the readily-available *meso*-tetraphenylporphyrin. We now report our exploitation of this weakly nucleophilic amine in generating reactive electrophilic and nucleophilic monofunctional porphyrins for attachment to side-chain extended α,ω-bis(methylamino) peptides. Polymers derived from the latter will be of use in MRI.

α,ω-Bis[benzyloxycarbonyl(methyl)amino] peptides with carboxylic acid derivatives and primary amines in side-chains of the same length were built up as shown in Scheme 1. *N*-(Benzyloxcarbonyl)sarcosine 1[9] was converted to its pentafluorophenyl (PFP) active ester 2† and this was added to a 20-fold excess of ethane-1,2-diamine to set up the protected sarcosine aminoethylamide 3† as the sequence inverting unit for the C-termini of the peptides.

Orthogonal protection of the α- and ε-amines of L-lysine 4 was required for elaboration of the peptide chain and of the side-chain. This was achieved by complexation with copper(II), selective acylation of the ε-amine with 2,2,2-trichloroethyl chloroformate, decomplexation and acylation of the α-amine with di-*tert*-butyl dicarbonate in a two-phase system, in a modification of the method of Yajima *et al.*[10] The resulting BocLys(Troc)OH 5† was converted to the PFP active ester 6,† prior to coupling with 3 to afford the fully orthogonally



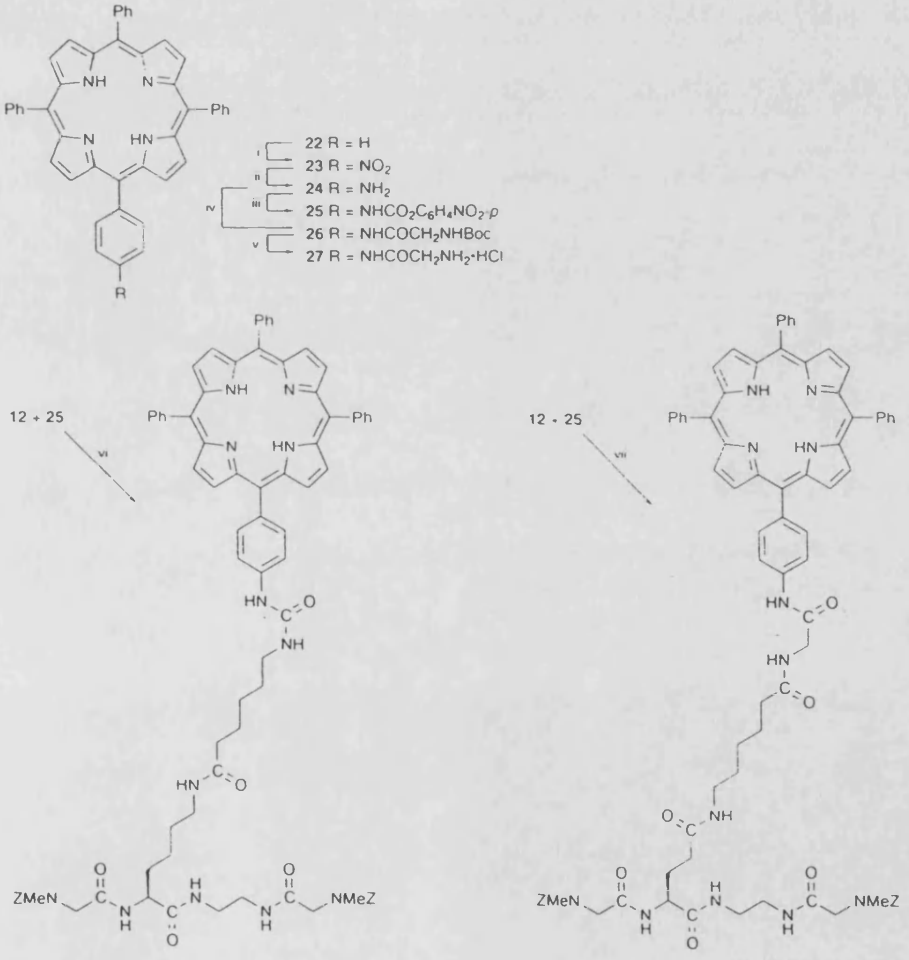

Scheme 1 Synthesis of extended sequence-inverted peptides 12 and 21. Troc = 2,2,2-trichloroethoxycarbonyl. *Reagents and conditions*:†† i, $C_6F_5OH$, DCC, EtOAc, 0 °C, 20 h, 90–95%; ii, $H_2NCH_2CH_2NH_2$ (20 × excess), $CH_2Cl_2$, 3 h, 82%; iii, $CuCO_3$, $H_2O$, reflux, 3 h, then TrocCl, $Na_2CO_3$, $H_2O$, 0 °C, 20 h; iv, $Na^+_3$ $EDTA^{2-}$, $H_2O$, reflux, 2 h; v, $Boc_2O$, $Et_3N$, $H_2O$, dioxan, 3 d, 58% from 4; vi, 3, $Pr^i_2NEt$, $CH_2Cl_2$, 85%; vii, HCl, $CH_2Cl_2$, 1 h, quant.; viii, 2, $Pr^i_2NEt$, DMAP, $CH_2Cl_2$, 4 d, 87%; ix, Zn, MeOH, reflux, 5 h, 83%; x, $BocNH(CH_2)_5CO_2C_6F_5$, $Pr^i_2NEt$, DMAP, $CH_2Cl_2$, 6 d, 55%; xi, $H_2N(CH_2)_5CO_2Me$·HCl, $Pr^i_2NEt$, DMAP, $CH_2Cl_2$, 7 d, 89%; xii, $H_2$, Pd/C, tetrahydrofuran, 3 h, quant; xiii, $N_2H_4$·$H_2O$, MeOH, 40 °C, 8 h, quant; xiv, $Bu^tONO$, DMF, THF, dioxan, HCl, −20 °C, 50 min, then $Pr^i_2NEt$, −60 °C (this solution was taken forward for reaction with 27, Scheme 2).

protected inverted-sequence peptide 7.† The peptide chain was completed by acidic removal of the Boc group, giving the salt 8,† and acylation with one further equivalent of 2. With the inverted sequence of the peptide now complete, attention was turned to extension of the length of the side-chain of 9.† The ε-amine 10† was revealed by selective reductive removal of the Troc group (zinc dust in methanol). Acylation with pentafluorophenyl 6-(*tert*-butoxycarbonylamino)hexanoate[11] gave the orthogonally protected peptide derivative 11.† Deprotection (HCl) afforded the target inverted-sequence peptide 12† with the extended primary amine side-chain for coupling with an appropriate monofunctional porphyrin electrophile.

In the assembly of a corresponding inverted-sequence peptide with an extended activated carboxylic acid side-chain, the extension was performed prior to construction of the peptide to avoid problems of formation of pyroglutamates. Acylation of the spacer unit, methyl 6-aminohexanoate, with Boc glutamic acid α-benzyl ester γ-PFP ester 13[12] gave the extended derivative 14.† The α-carboxylic acid 15† was revealed by selective hydrogenolysis of the benzyl ester. In a series of steps similar to those used for building the inverted-sequence lysine peptide, this carboxylic acid was activated as the PFP ester 16† and coupled with the sequence-inverting unit 3 to afford 17.† Again, selective acidolytic deprotection revealed the Glu α-amine 18. Coupling with 3 afforded the target inverted-sequence peptide 19† with the extended carboxylic acid side-chain protected as the methyl ester. This ester resisted selective hydrolysis under both basic and acidic conditions but succumbed to hydrazinolysis, giving the hydrazide 20.† From this, the acyl azide 21†‡ was formed by reaction with *tert*-butyl nitrite under acidic conditions, other nitrosating agents (*e.g.* sodium nitrite) being either inefficient or destructive. This provides an active acylating function for reaction with an appropriate porphyrin nucleophile.

The monoaminophenylporphyrin 24 was prepared in 46% overall yield in two steps from *meso*-tetraphenylporphyrin 22, in a modification of the method of Kruper *et al.* (Scheme 2).[8] The corresponding isocyanate would represent a potent electrophile for reaction with the extended lysine derivative 12 but 24 reacted slowly with phosgene, giving mainly the corresponding *N,N'*-bis(tetraphenylporphyrinyl)urea. However, the amine 24 was acylated smoothly§ by 4-nitrophenyl chloroformate, giving the carbamate 25, a synthon for the required isocyanate. Treatment of 25 under mildly basic conditions generated the isocyanate which coupled *in situ*§ with the extended lysine derivative 12, giving the protected porphyrinyl peptide derivative 28¶ in good yield.

The arylamine 24 was found to be a remarkably weak nucleophile, reacting with succinic anhydride only after a prolonged period at elevated temperature and not reacting with




Scheme 2 Activation of tetraphenylporphyrinamine 24 as an electrophile and as a nucleophile, coupling with extended sequence-inverted peptides 12 and 21. *Reagents and conditions*: †† i, fuming $HNO_3$, $CHCl_3$, 5 h, 55%; ii, $SnCl_2$, conc. aq. HCl, 80 °C, 2 h, 84%; iii, 4-nitrophenyl chloroformate, $Pr^i_2NEt$, $CHCl_3$, 20 h, then chromatography, 67%; iv, $BocGlyOC_6F_5$, $Pr^i_2NEt$, DMAP, $CHCl_3$, 46 h, 95%; v, HCl, $CH_2Cl_2$, 1 h, quant; vi, $Pr^i_2NEt$, DMAP, $CH_2Cl_2$, 20 h, 82%; vii, $Pr^i_2NEt$, DMAP, $CHCl_3$, 2 h, 58%.

peptide active esters or with the acyl azide **21**. Much greater nucleophilicity is required for efficient coupling with peptide derivatives under mild conditions. To introduce a primary aliphatic amine as a more potent nucleophile, the arylamine was acylated by treatment‖ with a two-fold excess of the PFP active ester of N-Boc-glycine at 40 °C, forming **26**. The primary aliphatic amine **27** was revealed by deprotection‖ with hydrogen chloride. This more reactive nucleophile then coupled efficiently‖ with the extended peptide derivative acyl azide **21**, giving the porphyrinyl peptide derivative **29**.**

The monoaminotetraphenylporphyrin **24** is thus demonstrated to be a readily accessible monofunctionalised porphyrin which can be converted straightforwardly into a reactive electrophile and a reactive unhindered nucleophile which should have general utility in controlled attachment of porphyrins to peptides, polymers and other molecules.

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## Footnotes

† All novel compounds were characterised by $^1$H NMR and by FAB MS and were shown to be pure by TLC. Target compounds and major intermediates were also characterised by high resolution FAB MS.

‡ Acyl azide **21** was prepared and used without purification, to avoid Curtius rearrangement.

§ *Method*: Compound **24** (5.70 g, 9.25 mmol) was stirred with 4-nitrophenyl chloroformate (1.86 g, 9.25 mmol) and $Pr^i_2NEt$ (1.19 g, 9.25 mmol) in $CHCl_3$ (50 cm$^3$) for 20 h. Chromatography gave **25** (4.85 g, 67%) as a purple glass. Compound **12** (385 mg, 530 μmol) and **25** (520 mg, 670 μmol) were stirred with $Pr^i_2NEt$ (205 mg, 1.6 mmol) and DMAP (10 mg) in $CH_2Cl_2$ (10 cm$^3$) for 20 h. Chromatography gave **28** (593 mg, 82%) as a purple glass.

¶ *Spectroscopic data* for **28**: δ ($CDCl_3$) −2.78 (2 H, s, porphyrin 21,23-$H_2$), 1.2–1.7 (10 H, m, Lys β, γ-$H_4$ + $NCH_2CH_2CH_2CH_2CH_2CO$), 2.12 (2 H, br, $CH_2CH_2CO$), 2.93 (3 H, s, $NCH_3$), 2.99 (3 H, s, $NCH_3$), 3.0–3.3 (8 H, Lys ε-$H_2$ + $NCH_2CH_2CH_2$ + $NCH_2CH_2N$), 3.77 (2 H, m, Sar-$H_2$), 3.86 (2 H, m, Sar'-$H_2$), 4.36 (1 H, m, Lys α-H), 5.06 (3 H, s) and 5.07 (1 H, s) (2 × $PhCH_2O$), 5.75 (1 H, br, NH), 5.83 (1 H, br, NH), 6.39 (1 H, br, NH), 6.57 (1 H, br, NH), 6.94 (1 H, br, NH), 7.24 (10 H, br s, 2 × benzyloxy Ph-$H_5$), 7.37 (1 H, br, NH), 7.69 (11 H, m, 3 × porphyrin-Ph 3,4,5-$H_3$ + porphyrin-$C_6H_4N$ 2,6-$H_2$), 8.06 (2 H, d, *J* 8.4 Hz, porphyrin-$C_6H_4N$ 3,5-$H_2$), 8.15 (6 H, m, 3 × porphyrin-Ph 2,6-$H_2$), 8.79 (2 H, d *J* 4.7 Hz, porphyrin 3,7-$H_2$), 8.82 (4 H, s, porphyrin 12,13,17,18-$H_4$), 8.87 (2 H, d, *J* 5.1 Hz, porphyrin 2,8-$H_2$); *m/z* (FAB) 1367.6384 (M + H) ($C_{81}H_{83}N_{12}O_9$ requires 1367.6406).

‖ *Method*: Compound **24** (5.00 g, 8.16 mmol) was stirred with Boc-$GlyOC_6F_5$ (5.58 g, 16.3 mmol), $Pr^i_2NEt$ (2.32 g, 18.0 mmol) and DMAP (50 mg) in $CHCl_3$ (100 cm$^3$) for 46 h at 40 °C. Chromatography gave **26** (6.00 g, 95%) as a purple glass. This compound (2.24 g, 2.9 mmol) was treated with excess HCl in $CH_2Cl_2$ (100 cm$^3$) for 1 h. The solvent and excess reagent were evaporated to give **27** (2.10 g, quantitative). *tert*-Butyl nitrite (0.22 cm$^3$) in THF (1.75 cm$^3$) was added to **21** (1.50 mg, 2.07 mmol) in DMF (3.0 cm$^3$) and HCl in 1,4-dioxan (4.0 mol dm$^{-3}$, 1.86 cm$^3$) at −20 °C. The mixture was stirred for 2 h. $Pr^i_2NEt$ (1.06 g) was added at −60 °C, followed by **27** (2.09 g, 2.9 mmol) and $Pr^i_2NEt$ (1.12 g, 18.7 mmol) in $CHCl_3$ (30 cm$^3$). The mixture was stirred for 2 h. Chromatography gave **29** (1.64 g, 58%) as a purple glass.

** *Spectroscopic data* for **29**: $\delta_H$ ($CDCl_3$) −2.75 (2 H, porphyrin 21,23-$H_2$), 0.89 (2 H, m, $NCH_2CH_2CH_2CH_2CH_2CO$), 1.25–1.65 (6 H, m, $NCH_2CH_2CH_2CH_2CH_2CO$ + Glu β-$H_2$), 1.9–2.4 (4 H, Glu γ-$H_2$ + $CH_2CH_2CH_2CO$), 3.0–3.1 (8 H, m, $NCH_2CH_2CH_2$ + 2 × $NCH_3$), 3.35 (4 H, br, $NCH_2CH_2N$), 3.85–4.05 (4 H, m, 2 × Sar-$H_2$), 4.15–4.25 (2 H, m, Gly-$H_2$), 4.42 (1 H, m, Glu α-H), 5.13 (4 H, br s, 2 × $PhCH_2O$), 7.25–7.33 (14 H, m, 4 × NH + 2 × benzyloxy Ph-$H_5$), 7.70–7.76 (11 H, m, 3 × porphyrin-Ph 3,4,5-$H_3$ + porphyrin-$C_6H_4N$ 2,6-$H_2$), 7.96 (2 H, m, 2 × NH), 8.12 (2 H, d, *J* 8.2 Hz, porphyrin-$C_6H_4N$ 3,5-$H_2$), 8.17–8.23 (6 H, m, 3 × porphyrin-Ph 2,6-$H_2$), 8.84 (8 H, br s, porphyrin 2,3,7,8,12,13,17,18-$H_8$); *m/z* (FAB) 1381.6184 (M + H) ($C_{81}H_{81}N_{12}O_{10}$ requires 1381.6199).

†† Reactions took place at ambient temperature, unless otherwise stated.

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